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THE JOURNAL  
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EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME THIRTY-FOUR  
WITH FIFTY-FOUR PLATES AND  
ONE HUNDRED AND SEVEN FIGURES  
IN THE TEXT



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## EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

### V. BACTERIUM PNEUMOSINTES AND CONCURRENT INFECTIONS.

BY PETER K. OLITSKY, M.D., AND FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 1 TO 3.

(Received for publication, March 1, 1921.)

In the preceding article<sup>1</sup> of this series, we described the anaerobic, filter-passing organism which has been cultivated by special methods from the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza, and from the lungs of rabbits and guinea pigs experimentally inoculated with these secretions. Earlier experiments had demonstrated the presence, in the nasopharyngeal secretions, of an active agent of peculiar character, distinguished from ordinary bacteria by its effects on the lungs and blood of experimental animals, by filter passage, and by resistance to glycerolation for a period of months.<sup>2,3</sup> Parallel experiments with the anaerobic organism disclosed a similarity of biologic properties and pathogenic effects sufficient to establish its identity with the active agent. We have, therefore, stated<sup>1</sup> that the pathogenic activity of the nasopharyngeal washings from early cases of uncomplicated epidemic influenza, as tested in our experimental animals, was due to the presence of the anaerobic, filter-passing organism which we have recovered from these secretions.

A peculiar and significant property of the active agent, in view of its origin, was its effect in reducing the resistance of the lung tissues of inoculated animals to accidental or experimental infection with bacteria of ordinary species—those bacteria for example which were so

<sup>1</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

<sup>2</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

<sup>3</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.

frequently found in concurrent or secondary pneumonias associated with epidemic influenza in man.<sup>4</sup>

If the active agent in influenzal secretions has been identified in the filter-passing organism, it follows that this organism should have the same property of reducing pulmonary resistance in experimental animals. It is the purpose of the present paper to describe our observations and experiments bearing on this point.

*Accidental Concurrent Infections with Ordinary Bacteria.*

Under the heading of accidental infections may be grouped those scattered experiments in which the intratracheal inoculation of a washed mass culture of the filter-passing organism was followed by a pulmonary infection with ordinary bacteria. We have already described such accidents after inoculation of the active agent from influenzal washings.<sup>4</sup> These experiences were less frequent in the later experiments with the filter passer, due to the avoidance of oral contamination after the catheter method first employed was discarded in favor of needle puncture of the exposed trachea.<sup>5</sup>

Nevertheless, ordinary bacteria were encountered in the lungs of six rabbits in five series of transmission experiments with cultures originally derived from a strain of the filter-passing organism obtained from a case in the first, or 1918-19, epidemic. Three other transmission series remained uncomplicated throughout. In these experiments the first passage was initiated with a culture of the filter passer. Subsequent passages were effected with suspensions of lung tissue from the preceding rabbits. Thus the organism was carried through as many as five rabbit passages, and subsequently recovered.

*Bacillus welchii* was recovered from two successive rabbit passages, the fourth and fifth, of one transmission series. *Bacillus coli*, *Bacillus leprosepticus*, *Staphylococcus aureus*, and a large Gram-positive bacillus were each recovered once (Table I). The occurrence of such an accidental infection caused the termination of the series.

The presence of a concurrent bacterial infection in these rabbits was usually indicated by aggravated symptoms and by prostration

<sup>4</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.

<sup>5</sup> All operations were performed under light ether anesthesia.

and death within 48 hours if the animal was not killed earlier. An intense injection or a purulent inflammation of the conjunctivæ developed. Loss of weight was marked. The blood picture showed a greater depression of the leucocytes, involving both the polymorphonuclear and the mononuclear cells than that occurring in the animals which were injected with *Bacterium pneumosintes*. In the rabbits in which *Bacillus coli* and the Gram-positive bacillus were encountered, the initial depression was followed by a polymorphonuclear leucocytosis.

TABLE I.

*Occurrence of Ordinary Bacteria in the Course of Transmission Experiments with B. pneumosintes.*

Generation of <i>B. pneumosintes</i> beginning the series.	No. of rabbit passages showing typical effects.	Rabbit passage showing secondary infection.	Kind of bacteria.	Pathological effect in rabbit.
Second.	5	Second (only in one of two rabbits).	<i>Staphylococcus aureus</i> .	Diffuse polymorphonuclear consolidation with abscesses.
Fourth.	2	Second.	<i>B. coli</i> .	Abscess of lungs.
"	2	" (only in one of two rabbits).	Large Gram-positive aerobic bacillus.	" " "
Eighth.	5	Fourth. Fifth.	<i>B. welchii</i> . " "	Bronchopneumonia. "
Ninth.	4	Fourth.	" <i>leptisepticus</i> .	Purulent bronchitis; patchy pneumonia.

At autopsy the familiar pulmonary lesions—hemorrhagic edema and emphysema without consolidation or pleuritis—were complicated or masked by other lesions attributable to the ordinary bacteria involved. A diffuse polymorphonuclear exudation was accompanied by patchy or lobar consolidation, localized small abscesses, and necrosis of the vascular endothelium with thrombus formation. A purulent bronchitis resulted from the *leptisepticus* infection. From these lesions the invading organisms were cultivated and identified.

Thus the course of accidental bacterial infection in rabbits inoculated with the filter passer closely paralleled the findings in similar infections accompanying the active agent of the earlier experiments. A more exact basis of comparison is afforded by parallel experiments with the active agent and the filter-passing organism in which concurrent or secondary bacterial infections were experimentally induced.

### *Experimental Concurrent Infections.*

With the exception of *Staphylococcus aureus*, the accidental invaders in our experiments do not belong to the group of organisms commonly found in concurrent or secondary pneumonias so frequently associated with epidemic influenza in man. For the production of experimental concurrent infections we therefore chose two organisms as examples of the frequent inhabitants of the nasopharynx which have been recovered from many postinfluenzal pneumonias, *Bacillus pfeifferi* and a Type IV pneumococcus.

We have already described<sup>4</sup> the results of intratracheal or intravenous injection of these organisms alone in the small doses employed in the following experiments. Their effects were transient and differed essentially from the equally transient effects of the influenzal active agent. It was the combined action of bacteria and active agent in the same rabbit which produced the fatal pneumonias in the experimental animals and led us to point out the significant similarity of these pneumonias to those associated with epidemic influenza in man. With these earlier experiments as a basis of comparison we were now ready to study the effects of the ordinary bacteria in combination with *Bacterium pneumosintes*.

Rabbits were first inoculated intratracheally with the cultivable bodies and then received suitable doses of the chosen bacteria either intratracheally or by an ear vein.

The following protocols illustrate the results of the injection of cultivable bodies and ordinary bacteria by the intratracheal route.

*Protocol 1.*<sup>6</sup> *B. pneumosintes* and *Pneumococci*.—Nov. 17, 1920. A rabbit whose normal temperature was 38.9°C., total leucocytes 11,000, of which 4,620 were mononuclears, was inoculated intratracheally with the washed sediment of a

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<sup>6</sup> Only typical protocols of a number of similar experiments are presented.

fourth generation mass culture<sup>7</sup> of cultivable bodies originally derived from the 1918-19 epidemic. Nov. 18. Temperature 39.3°C., total leucocytes 8,600, of which 1,892 were mononuclears. Inoculated intratracheally with one loopful of a 48 hour growth of Type IV pneumococcus on a standard agar slant. Nov. 20. Temperature 40°C., leucocytes 6,000, of which 2,400 were mononuclears (Text-fig. 1). Killed. The lungs showed lobar consolidation (red hepatization) of the right upper and lower lobes and a small patch of consolidation in the left upper lobe. The lesion in the left lower lobe consisted of edema, emphysema, and a number of small hemorrhages. Film preparations of the consolidated area showed 80 per cent polymorphonuclear cells and a few pneumococci. Aerobic cultures yielded *Pneumococcus* Type IV.

Control rabbits were separately injected with the same doses of *B. pneumosintes* and the pneumococcus. In the first instance the injection was followed in 48 hours by a fall in the total leucocyte count, mainly due to a drop in the mononuclears. The rabbit was killed. At autopsy the lungs showed a typical hemorrhagic edema and emphysema without consolidation. Aerobic cultures remained sterile. Anaerobic cultures yielded *B. pneumosintes* in pure culture.

The second control rabbit, injected intratracheally with one loopful of pneumococcus culture, was killed after a similar interval. The lungs showed no lesion. Aerobic cultures were free from growth.

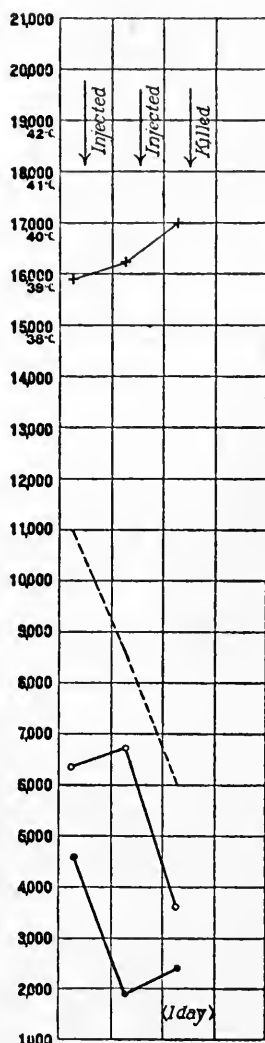
*Protocol 2. B. pneumosintes and B. Pfeifferi.*—Dec. 11, 1920. A rabbit whose normal temperature was 39.2°C. and total leucocytes 7,000, of which 3,850 were mononuclears, was inoculated with the washed sediment of a seventh generation mass culture of cultivable bodies originally derived from the first, or 1918-19, epidemic. Dec. 12. Temperature 39.5°C. Total leucocytes 4,600, of which 794 were mononuclears. Inoculated intratracheally with the 24 hour growth on a blood agar slant of *B. Pfeifferi*. Dec. 13. Temperature 39.5°C. Total leucocytes 10,000, of which 2,500 were mononuclears (Text-fig. 2). Killed. The lungs showed pneumonic consolidation of the right and left upper lobes, and edema, emphysema, and patchy hemorrhages in the other lobes. The diffuse polymorphonuclear exudation in the consolidated areas is shown in Fig. 1. Aerobic cultures yielded *B. Pfeifferi*.

A control rabbit, inoculated intratracheally with the same dose of *B. pneumosintes* showed the clinical and pathological effects regarded as typical and already described at length<sup>1</sup> (Fig. 2).

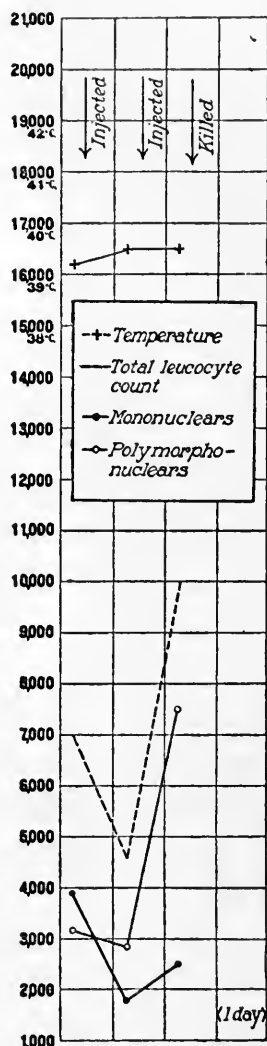
A second control rabbit was injected intratracheally with the washed sediment from an uninoculated control of the mass culture medium, and on the following day with the same dose of *B. Pfeifferi* that was given to the experimental animal. This control rabbit was killed 24 hours later. The lungs showed no lesions. Aerobic cultures yielded no growth.

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<sup>7</sup> The dose and preparation of the growth in mass culture for inoculation are described in another paper.<sup>1</sup>



TEXT FIG. 1.



TEXT FIG. 2.

TEXT-FIG. 1. Effect on blood count and temperature (Protocol 1). The first intratracheal injection consisted of the cultivable bodies and was followed by a rise in temperature and depression of the total leucocytic count, caused by a deficiency of mononuclears. The second intratracheal injection, of pneumococci, caused no essential change in temperature or blood count.

TEXT-FIG. 2. Effect on blood count and temperature (Protocol 2). The first intratracheal injection consisted of the cultivable bodies and was followed by a rise in temperature and depression of the total leucocytic count, caused by a deficiency of mononuclears. The second intratracheal injection, of *B. Pfeifferi*, caused a polymorphonucleosis.



The injection of cultures of the cultivable bodies, followed by sub-infective doses of pneumococci or Pfeiffer's bacilli, produced a lobar or bronchopneumonic consolidation of the lungs with polynuclear exudation, combined with the hemorrhagic edema and emphysema typical of the *Bacterium pneumosintes* and the influenzal active agent.

Cultures from the consolidated areas yielded profuse growths of pneumococci or *Bacillus pfeifferi*. Control rabbits injected with the cultivable bodies alone showed only the hemorrhagic edema and the emphysema, without consolidation (or polymorphonuclear cell invasion). Control rabbits injected with the ordinary bacteria, in the small doses employed, showed only a transient polymorphonuclear leucocytosis and no visible lung lesions.

These experiments, therefore, gave results similar to those observed after the intratracheal injection of the active influenzal agent and *Bacillus pfeifferi* or a pneumococcus.

The intratracheal injection of both organisms imitated, in a manner, the probable mode of infection in man. In a second series of experiments, as in the former experiments with the influenzal agent, the intravenous route for the injection of the ordinary bacteria was chosen as a more severe test of concomitant action, even though the blood stream may not be the portal of entry of the lungs in postinfluenzal pneumonias in man.

A series of rabbits was injected intratracheally with a third generation culture of the cultivable bodies, originally derived from the nasopharyngeal secretions of a case in the first epidemic (1918-19). 24 hours later they were given, by ear vein, small doses of a Type IV pneumococcus or of Pfeiffer's bacillus which proved subinfective for control animals.

Under the conditions of the experiment the ordinary bacteria showed a selective localization in the lung tissues where they set up an active infection resulting in more or less extensive lobar or bronchial consolidation, with profuse polymorphonuclear exudation and fibrin formation with necrosis of the vessel walls and thrombus production. The rest of the lung tissue showed the hemorrhagic edema and the emphysema characteristic of infection with *Bacterium pneumosintes* alone. Type IV pneumococci or Pfeiffer's bacilli were recovered from the consolidated areas.

These observations on the results of experimental concurrent infection with the cultivable bodies and ordinary bacteria closely parallel those already described<sup>1</sup> as the result of similar experiments with the active agent and the corresponding ordinary bacteria.

They demonstrate that *Bacterium pneumosintes* possesses the same peculiar property of lowering the threshold of resistance of the pulmonary structure to infection with ordinary bacteria. As a result subinfective doses of Type IV pneumococci and of Pfeiffer's bacilli, for example, become infective and invade the vulnerable tissue, with the establishment of such reactions as are typical of postinfluenzal pneumonia in man. Thus additional proof is afforded of the identity of the active agent of the nasopharyngeal secretions in influenza and the cultivable bodies derived from the same source, and a further parallel is drawn between the accidental or experimental production of concurrent or secondary bacterial pneumonias in animals and the frequent occurrence of similar postinfluenzal pneumonias in man.

#### SUMMARY.

During the course of animal experiments with the anaerobic filter-passing organisms cultivated from epidemic influenzal sources, certain pulmonary infections with ordinary bacteria have been observed. The experiments also have shown that the lungs of animals infected with *Bacterium pneumosintes* are less resistant than normal lungs to infection with ordinary bacteria. The demonstration of this fact invites a comparison of the course of these experimental bacterial infections with the sequence of postinfluenzal pneumonias attributable to similar organisms in man.

These observations furnish additional proof of the identity of *Bacterium pneumosintes* and the active agent derived from the nasopharyngeal secretions of patients in the early hours of epidemic influenza.

## EXPLANATION OF PLATES.

## PLATE 1.

FIG. 1. Microscopic lesions in the lungs of a rabbit described in Protocol 2, injected intratracheally with *B. pneumosintes* followed by similar inoculation in 24 hours with *B. Pfeifferi*. The diffuse polymorphonuclear exudation is noteworthy. Compare with Fig. 2.  $\times 95$ .

## PLATE 2.

FIG. 2. Microscopic lesions in the lungs of a rabbit described in Protocol 2, and injected intratracheally with the cultivable bodies alone. The hemorrhagic edema and emphysema are noteworthy.  $\times 95$ .

## PLATE 3.

FIG. 3. Gross lesions in the lungs of a rabbit injected intratracheally with the cultivable bodies, and 24 hours later, intravenously with pneumococci. The hemorrhagic consolidation of the left lung, and the hemorrhagic edema and emphysema of the right are noteworthy. Natural size.

FIG. 4. Gross lesions in the lungs of a rabbit injected intratracheally with the cultivable bodies, and 24 hours later, intravenously with *B. Pfeifferi*. The hemorrhagic and patchy consolidation of the left lung, and the hemorrhagic edema and emphysema of the right are shown. Natural size.



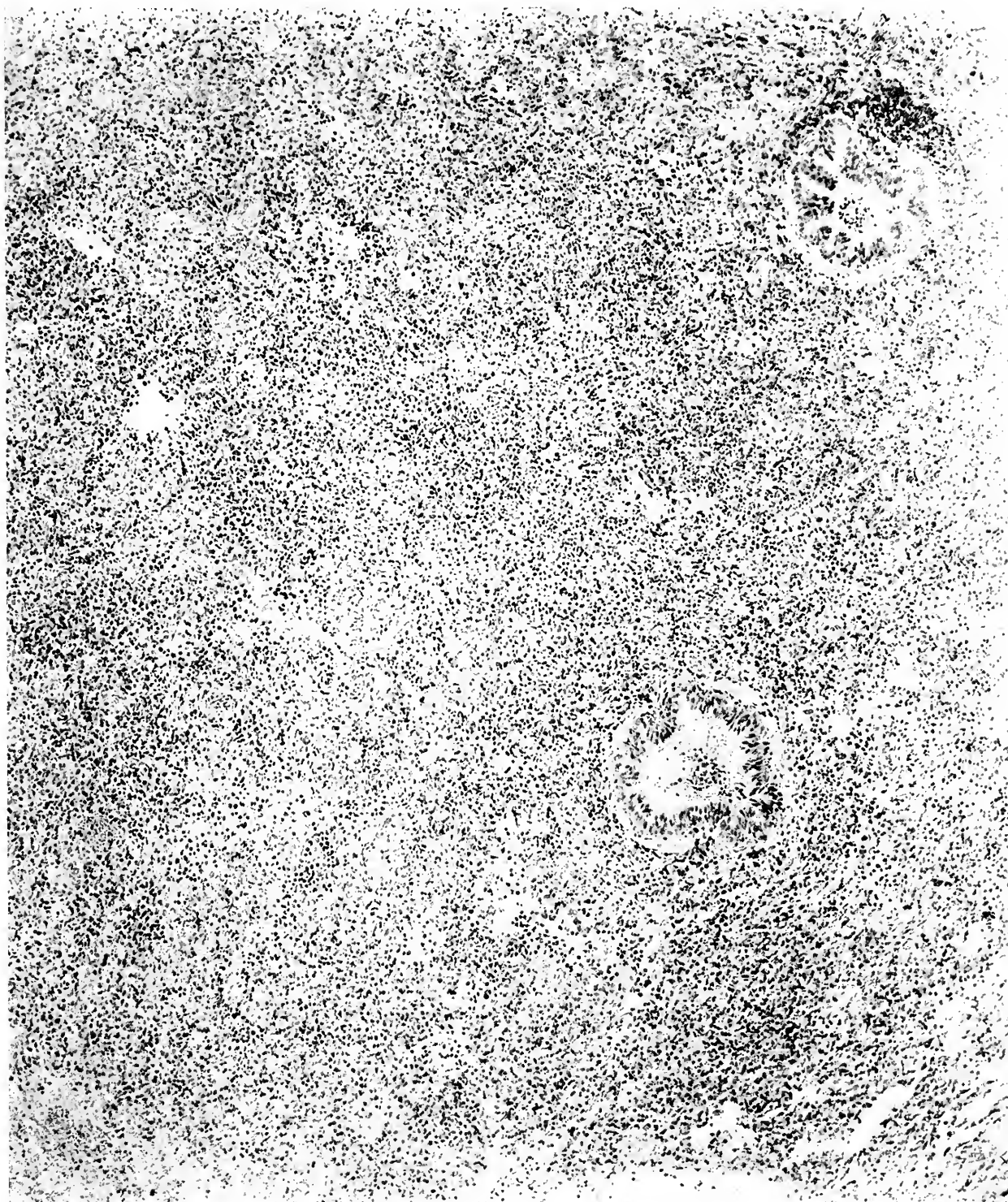


FIG. 1.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. V.)



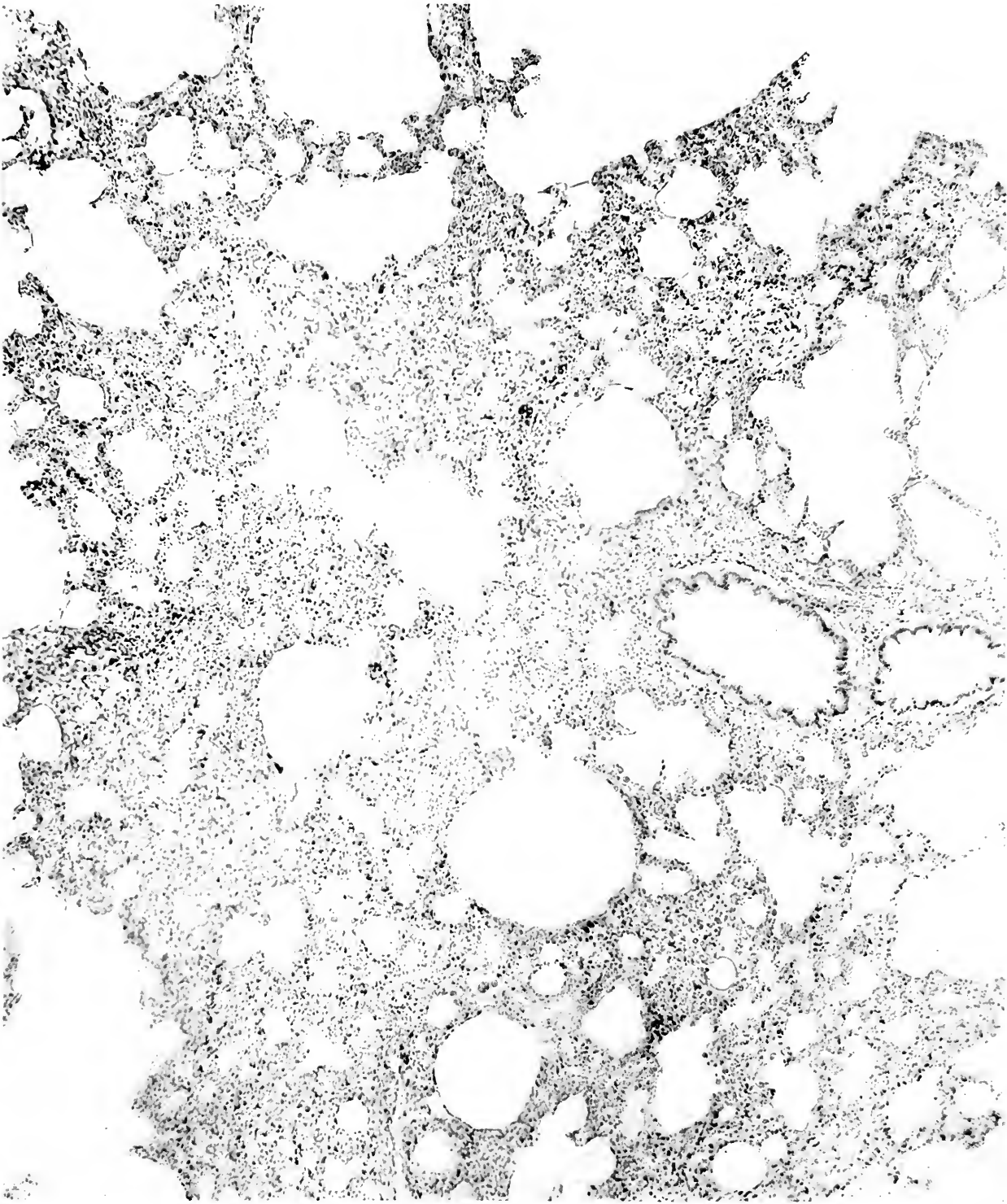


FIG. 2.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. V.)







FIG. 3.



FIG. 4.



# REACTIONS OF THE NASAL CAVITY AND POSTNASAL SPACE TO CHILLING OF THE BODY SURFACE.

## I. VASOMOTOR REACTIONS.

BY STUART MUDD, M.D., ALFRED GOLDMAN, M.D., AND SAMUEL B. GRANT, M.D.

*(From the Department of Pathology of Washington University Medical School, St. Louis, and the Laboratories of Biophysics of the Cancer Commission of Harvard University, Boston.)*

(Received for publication, March 3, 1921.)

Chilling of the body surface has been shown in earlier communications<sup>1,2</sup> to cause reflex vasoconstriction and ischemia in the mucous membranes of the palate, palatine tonsils, and pharynx. The present study demonstrates a like reflex diminution in the blood supply of the nasal cavity and postnasal space (nasopharynx).

In the postnasal space the reaction is closely similar to that previously described for the oropharynx; with chilling of the body surface the temperature of the nasopharyngeal mucosa has fallen typically between 1° and 2°C.; on rewarming the subject, mucosa temperature rises, indicating return toward normal of the blood supply, but, typically, under the conditions of our experiments, recovery is not quite complete even after an interval at least as long as  $\frac{1}{2}$  hour after wrapping.

In the nasal cavity the reactions are qualitatively similar but quantitatively much more striking; with cutaneous chilling the temperature depression of the nasal mucosa surface has been found in some instances to be more than 6°C. With rewarming, recovery has always been sharp, usually stopping somewhat below control level, but sometimes rising above it in this region notorious for its erratic variations in vasomotor state.

<sup>1</sup> Mudd, S., and Grant, S. B., *J. Med. Research*, 1919, xl, 53.

<sup>2</sup> Grant, S. B., Mudd, S., and Goldman, A., *J. Exp. Med.*, 1920, xxxii, 87.

The threshold of the vasoconstrictor reflex to the nasal and nasopharyngeal mucosa has been found to be lower than that to the skin of the forehead. Merely unwrapping the subject, in the cool room, temperature 14–18°C., in a number of instances caused depression of mucosa temperature without affecting that of the skin.

Profuse discharge of clear mucus, both from the side of the nose directly irritated and from the opposite side, although more abundantly from the former, occurred during most of those experiments in which the thermopile wires used for measuring surface temperature were introduced into the nasal cavity. This rhinorrhea was little if at all affected by the diminution of blood supply and shrinkage of the nasal mucous membrane which occurred in reflex response to chilling of the body surface.

Discharge from the nose has been at most a rare occurrence in experiments in which the nasal mucosa was not directly irritated.

### *Methods.*

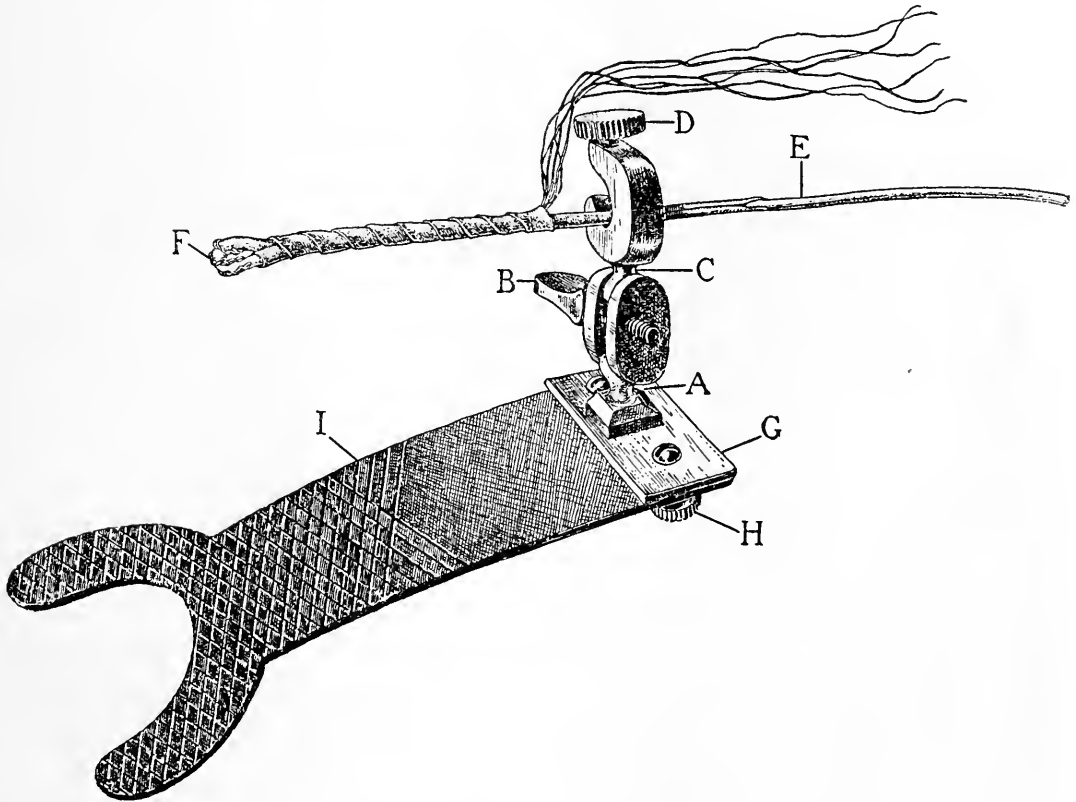
The methods used differed only in detail from those of the earlier papers. The vasomotor reactions were determined as before by following with thermopiles the temperature changes, synchronously, at the surface of the skin of the forehead, and of the particular mucous membrane site under consideration. A fall in surface temperature under the conditions of the experiments indicates vasoconstriction and diminished blood supply, a rise vasodilation.

Respiration as before was controlled by thoracic and abdominal pneumographs and a metronome. Breathing in the present series was through the nose with lips closed, 14 respirations per minute.

Chilling in many of the present experiments was performed in two stages; first the subject's wraps were removed in the cool room; later an electric fan was turned on and directed against the lumbar region of his back. When the fan was turned off, the wraps were replaced at the same time; rewarming was considerably more satisfactory than heretofore as two extra blankets were used for rewarming.

The thermopile tips were applied by means of No. 12 or 15 galvanized iron wire carriers whose applying ends were so shaped as to conform to the contour of the particular skin or mucous membrane surface in apposition with which the thermopile tips were to be held.

In the nasal cavity, the wire carrier, or applicator, was maintained in position by a special applicator holder. A metal spheroid (Text-fig. 1, *A*), attached to a metal plate, *G*, is connected by metal plates bearing sockets and closed by a set screw, *B*, with a second spheroid, *C*, thus making a double ball and socket joint. The spheroid, *C*, is



TEXT-FIG. 1. Nasal applicator holder with applicator and unknown temperature end of thermopile in position. *A*, metal spheroid mounted on metal plate, *G*. *B*, set screw for tightening lateral plates, bearing sockets, upon *A* and second spheroid, *C*. *D*, thumb screw for holding applicator, *E*, in place in groove in metal crescent continuous with *C*. *F*, applicator tip bearing the three insulated thermal junctions. *H*, one of two thumb screws attaching metal plate *G* to *I*, a fiber-board plate held between the subject's teeth.

continuous with a metal crescent into whose grooved bottom the wire applicator, *E*, fits and is held in the position desired by the thumb screw, *D*. The thermopile, with insulated tips at *F*, is bound to the applicator with adhesive tape.

The whole device is attached by screws fastened by two thumb screws, one of which is shown at *H*, to a plate, *I*, so shaped as to fit

firmly between the subject's teeth. This supporting plate is of fiber board, 1.68 mm. thick; although maintaining its shape quite well, it allows of a certain amount of molding by firm pressure. The plate, *I*, is crossed-hatched with a saw to facilitate gripping by the tips of the teeth. Making plates *I* and *G* detachable permits of having a separate fiber board plate for each subject.

The sites studied in the nose, because of the difficulty of making certain application farther back, were all in the anterior half of the cavity. The nasal septum, inferior and middle meatus, and inferior and middle turbinates were studied, the last at about the midportion of its inferior border at a point 5 cm. from the opening of the nostril. Since skin, anterior half of the nasal cavity, postnasal space, oropharynx, tonsils, and palate have now all been shown to exhibit reflex vasoconstriction with diminished blood supply on chilling of the body surface, and vasodilation on rewarming, it seems safe to assume that the posterior half of the nasal cavity does likewise.

For the postnasal space, no applicator holder was needed. The carriers were simply improved models of the original nasopharyngeal applicator shown in an earlier paper.<sup>3</sup>

The applying ends of the applicators of the present series held the thermopile tips against the posterior nasopharyngeal mucosa approximately 2.8, 3.3, and 3.7 cm., respectively, above the posterior margin of the soft palate when in the position of the experiments; *i.e.*, with mouth closed and nose breathing. The applicator with the 3.3 cm. vertical arm was the one chiefly used. As far as we could estimate from specimens in the anatomical museum, this must have carried the tips in most of the subjects as high as the upper half of the posterior nares, in some close to the roof of the nasopharyngeal vault.

For the skin, an applicator somewhat smaller and flatter than the one originally described<sup>4</sup> was used. Skin application was made upon the forehead in all instances.

The subjects of the present series were 3rd and 4th year medical students or recent graduates in medicine. We were fortunate in being able to include men of Aryan, Semitic, and one of Mongolian extraction. No racial differences in the vasomotor reactions were found.

<sup>3</sup> Mudd and Grant,<sup>1</sup> Fig. 1, *E*, p. 57.

<sup>4</sup> Mudd and Grant,<sup>1</sup> Fig. 1, *A* and *A'*, p. 57.

*Direct Cooling of the Skin by Air Currents.*

Although the sites of application of the thermopiles have been protected from the direct draft of the fan, minor currents and eddies have necessarily been set up by the fan in the small, closed experimental room, and the direct cooling effect of these upon the exposed skin could not be eliminated. This direct cooling, as closely as we have been able to estimate it, probably amounted usually to between one-third and one-half of the observed skin temperature fall. The curves both of this and of the previous series should be studied with this correction in mind.

On the other hand, it is obvious that in experiments in which the mouth was closed and the applicator upon the pharyngeal wall, as in the present series, currents in the room could not have entered at all into the depression of mucous membrane temperature. Similarly, with the applicator in the nasal cavity and nose breathing, again as in the present series, or on the palate or pharyngeal wall, even with the mouth open, direct cooling by air currents in the room could have entered but slightly or not at all into the observed mucous membrane temperature fall. The effect of the air currents, then, has been merely to make appear less striking in comparison with those of the skin the vasoconstrictor reflexes of the mucous membrane blood vessels with chilling of the body surface.

An experiment illustrating the direct cooling of the skin by secondary currents and eddies from the fan is given below.

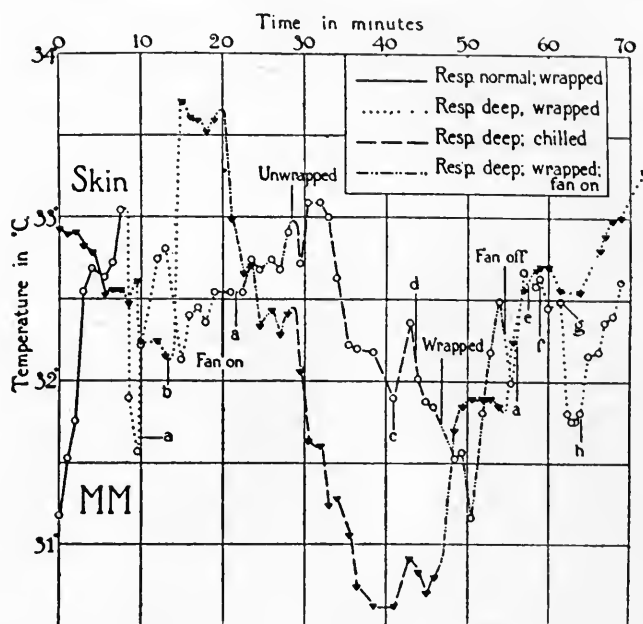
*Experiment 1. Control.*—Subject 1, F. J. C. July 19, 1920, 3.28 to 4.40 p.m. Application on hard palate, near posterior margin, just to right of midline. Breathing through open mouth. Room temperature about 19°C.

Turning the fan on the back of the subject at 0:20,<sup>5</sup> without removing the heavy wraps, depressed the temperature of the thermopile on the forehead by 1.3°C., without apparent effect upon the mucous membrane (Text-fig. 2). Unwrapping was followed by a further fall of 1.7°C. in skin temperature, one of 1.05° in that of the hard palate. After rewrapping, skin and mucosa temperatures returned to a little below their respective levels of before unwrapping. Turning the fan off, while without obvious effect upon mucosa temperature, was followed by return of that of the skin almost to control level.

In this experiment, therefore, there is no evidence of direct cooling of the mucosa of the hard palate by secondary currents from the electric fan; on the other hand, with the skin of the forehead, of

<sup>5</sup> 0:20, 0:21, etc., indicate the time after the beginning of the experiment; i.e., 0:20 indicates 20 minutes after the beginning of the experiment, 0:21, 21 minutes, etc.

the total temperature depression of  $3^{\circ}\text{C}$ . about  $1.3^{\circ}$  (43 per cent) was apparently due to direct cooling and  $1.7^{\circ}$  to loss of blood supply through reflex vasoconstriction.



TEXT-FIG. 2. Reflex reactions to chilling and rewarming; direct cooling effect of air currents on skin. Temperatures of skin of forehead and mucous membrane (MM) of hard palate, Experiment 1. *a*, respiration shallower than when first deepened; *b*, skin thermopile readjusted; *c*, subject laughed; *d*, no shivering as yet; *e*, respiration deepened; *f*, subject thinks respiration as deep as during chilling; *g*, subject forces respiration as hard as he can; *h*, end of maximal respiration.

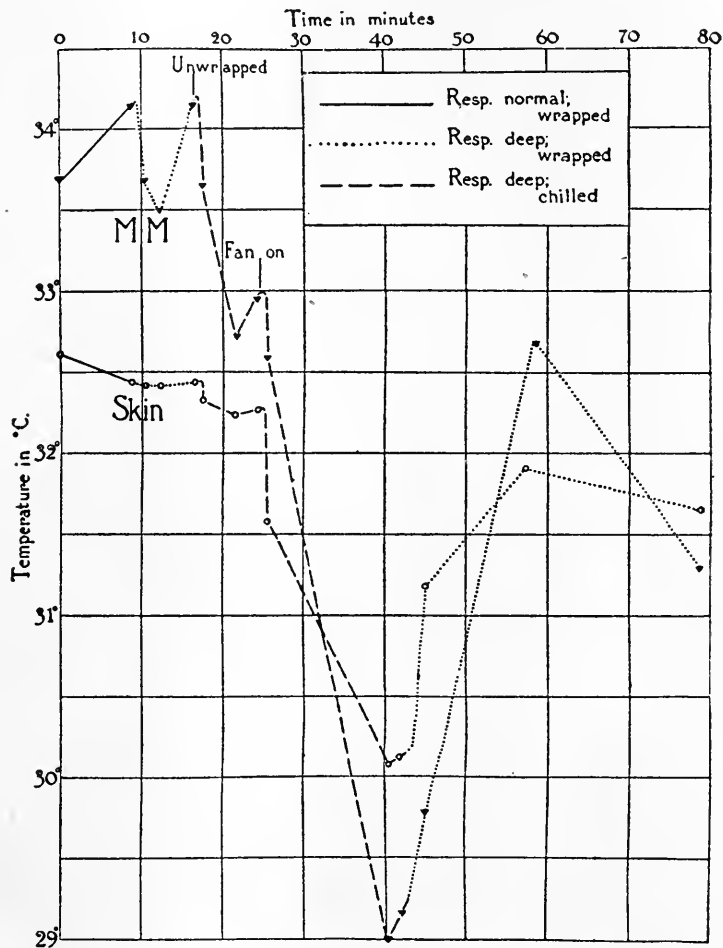
#### *Vasomotor Reactions in the Nasal Cavity.*

*Composite Graph.*—The characteristic responses of the mucous membrane of the nasal cavity to chilling of the body surface are shown in Text-fig. 3,<sup>6</sup> a composite graph of seven experiments. Application was made upon the right and left sides of the nasal septum, the right inferior turbinate body, the left inferior turbinate (twice), and in the left middle meatus (twice). The values averaged and plotted were the first and last readings of the experiments, the readings immediately before and after each change of conditions, and the points of maximum

<sup>6</sup> Text-figs. 3 and 4 were used to illustrate a brief summary of the intranasal part of the present study which has recently been published.<sup>22</sup>



response to changed conditions. The point of maximum depression with chilling on the mucous membrane curve is plotted separately, as is the minimal point in the skin curve, instead of, as heretofore, the minimal mucous membrane value only, with synchronous skin value. Similarly, after rewarming, the average maximum skin value and the average maximum mucous membrane value are given separately, and the synchronous point on the other curve in each case is omitted.



TEXT-FIG. 3. Nasal reactions to chilling and rewarming. Temperatures of skin and mucous membranes of nasal cavity; composite graphs of Experiments 2, 3, 4, 6, 7, 8, and 9.

Depression of mucous membrane temperature with deepened respiration was only  $0.65^{\circ}\text{C}.$ , and this was transient. Unwrapping the subject depressed mucous membrane temperature  $1.4^{\circ}$ , but skin temper-

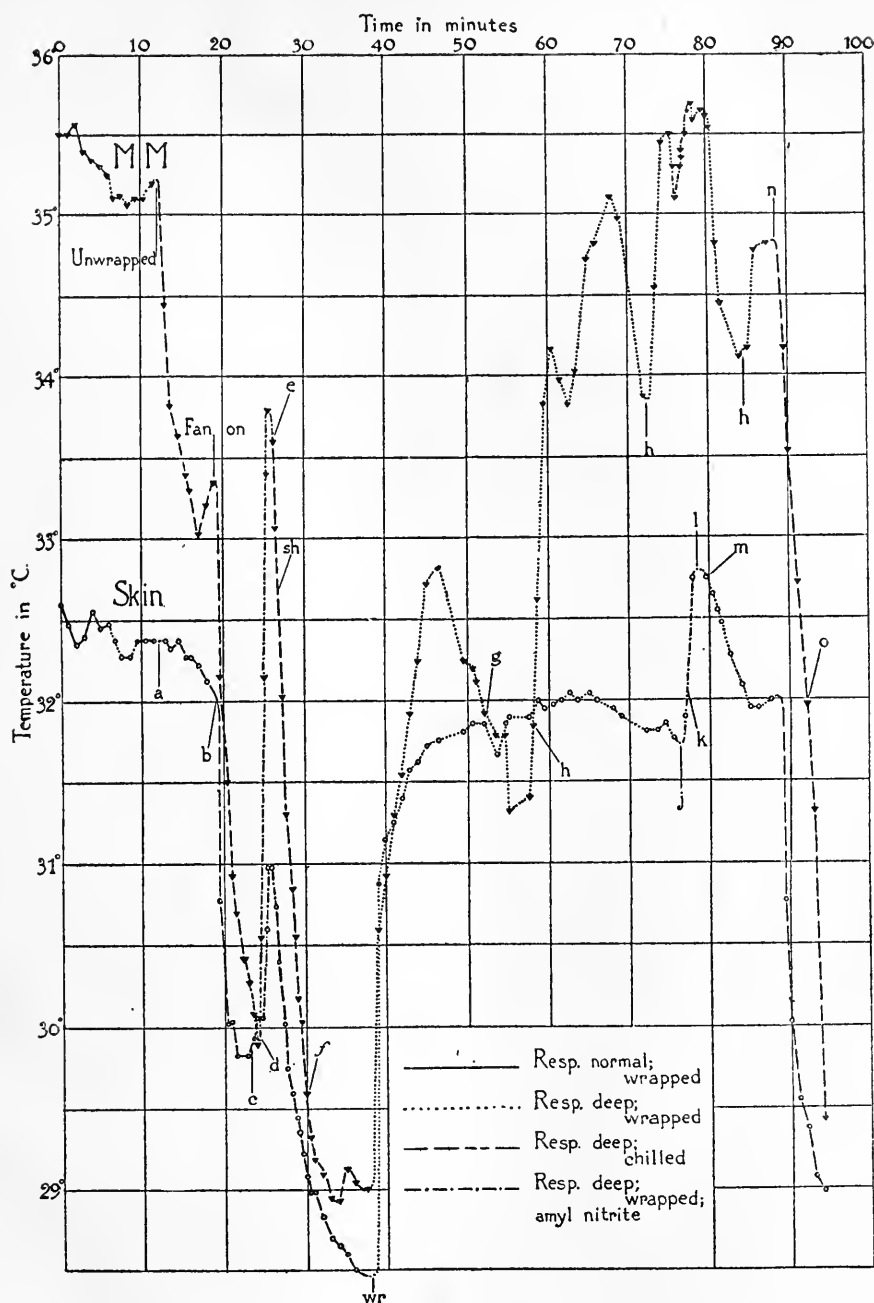
ature only  $0.2^{\circ}$ .<sup>7</sup> When the fan was turned on the subjects' backs, nasal mucosa and skin temperatures fell together, the former  $3.95^{\circ}$ , the latter  $2.2^{\circ}$ . The total mucous membrane temperature fall with chilling was  $5.15^{\circ}\text{C.}$ , a very striking result; that of the skin was  $2.4^{\circ}$ . The average maximum mucous membrane recovery with rewarming was  $3.7^{\circ}$  (72 per cent), that of the skin,  $1.8^{\circ}$  (75 per cent).<sup>8</sup>

*Experiment 2.*—Subject 2, A. G. June 23, 1920, 1.14 to 2.48 p.m. Application on anterior end of left lower turbinate body. Room temperature  $16\text{--}17^{\circ}\text{C.}$

Text-fig. 4<sup>6</sup> illustrates in an individual experiment the reactions brought out in the composite. With unwrapping at 0:12 the skin temperature is not depressed for 2.5 minutes; the mucous membrane temperature in the same interval falls  $1.6^{\circ}\text{C.}$  The pronounced drop in both mucous membrane and skin curves with fan on is interrupted by a sharp rise following amyl nitrite administration at 0:23.25, amounting, in the case of the mucosa, to  $3.9^{\circ}\text{C.}$ , in that of the skin to  $1.1^{\circ}\text{C.}$  After rewarming, the mucous membrane temperature in this experiment slightly more than regained its level of before chilling (in ten of the twelve intranasal experiments it remained somewhat depressed). Inhalation of amyl nitrite in this flushed condition of the mucous membrane resulted in a momentary depression of  $0.2^{\circ}$ , followed by a rise of  $0.6^{\circ}$ . Skin temperature rose  $1^{\circ}\text{C.}$ , approximately the same as before. The experiment ends with a profound vasoconstriction of mucous membrane and skin vessels incident to a second chilling with the fan.

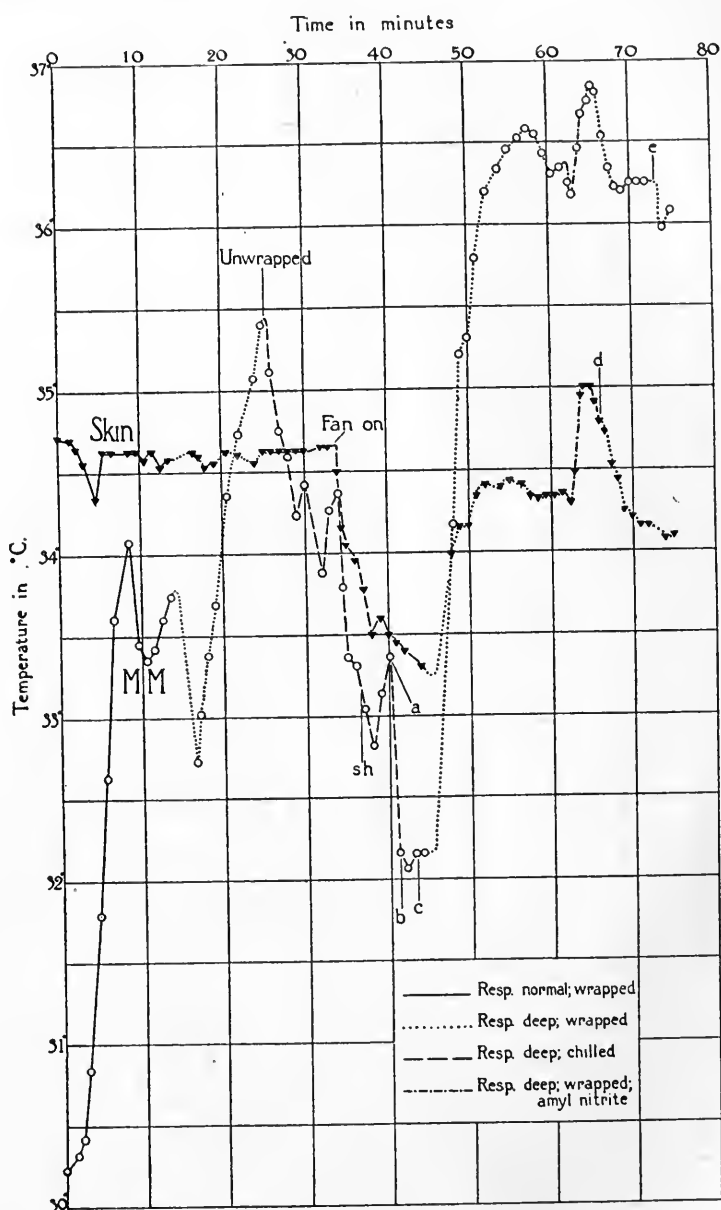
<sup>7</sup> Temperature values are calculated to one-hundredth degree as before, but in this paper are set down only to the nearest tenth (or twentieth when the value falls half-way between), since the accuracy of the method really does not warrant greater precision of statement than tenths of a degree.

<sup>8</sup> The fall in the mucous membrane temperature curve over the last 20 minutes is somewhat deceptive, and follows necessarily from the method of selecting the points for the composite graph. The points of maximum recovery are selected because they are maximal; the final points are merely those with which the experiment happens to end. In this graph a tendency of the mucous membrane applicator to slip forward, slightly out of position, probably contributed also to the apparent terminal temperature fall.



TEXT-FIG. 4. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of anterior end of left lower turbinate body, Experiment 2. *a*, subject unwrapped; *b*, fan on; *c*, begins to inhale amyl nitrite; *d*, face has begun to flush; *e*, flush fading; stops inhaling amyl nitrite; *sh*, shivers; *f*, shivering; *wr*, fan off, wrapped (two extra blankets added); *g*, mucous membrane applicator feels all right to subject; *h*, subject readjusts applicator so that he can feel it pressing against turbinate; *j*, starts inhaling amyl nitrite; respiration much increased; *k*, skin flushed; *l*, stops amyl nitrite; *m*, flush fading; *n*, unwrapped; fan on; *o*, shivering hard.

*Experiment 3.*—Subject 3, M. F. W. June 25, 1920, 12 m. to 1.37 p.m. Application in left middle meatus, 3.6 cm. from opening of nostril. Room temperature 16.5–18°C.



TEXT-FIG. 5. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of left middle meatus, Experiment 3. *sh*, begins shivering; *a*, not shivering; *b*, shivering; *c*, shivering hard; *d*, skin flush fading; *e*, left nose partially occluded; snuffs back mucus.

In Text-fig. 5 the marked upward trend of mucous membrane temperature except where interrupted momentarily by the deepening of

respiration at 0:14 and again through the duration of the chilling, suggests one of the spontaneous variations in vasomotor state recognized as of frequent occurrence in the nasal mucous membrane, but little understood. Unwrapping the subject was without effect on the skin temperature but changed the rise in mucosa temperature into a fall of  $1.5^{\circ}\text{C}$ . The total fall in mucous membrane temperature,  $3.3^{\circ}$ , was with one exception, the smallest obtained in twelve intranasal experiments.

*Experiment 4.*—Subject 4, S. B. G. June 24, 1920, 9.19 to 10.36 a.m. Application near anterior end of left lower turbinate. Room temperature about  $14^{\circ}\text{C}$ .

In Experiment 4, even at the low room temperature of  $14^{\circ}\text{C}$ ., unwrapping was followed by only a very slight skin temperature fall,  $0.2^{\circ}$ , while mucous membrane temperature was depressed  $2^{\circ}\text{C}$ . The total mucous membrane fall in this experiment was the largest observed,  $6.5^{\circ}$ ; the maximum recovery after wrapping was  $4.65^{\circ}$ , an incomplete return which seems to be more characteristic than the hyperemia after wrapping in the two previous experiments. Of the twelve nasal experiments, recovery was incomplete in ten, more than complete in two.

Amyl nitrite interposed a small rise in the mucous membrane temperature fall from chilling.

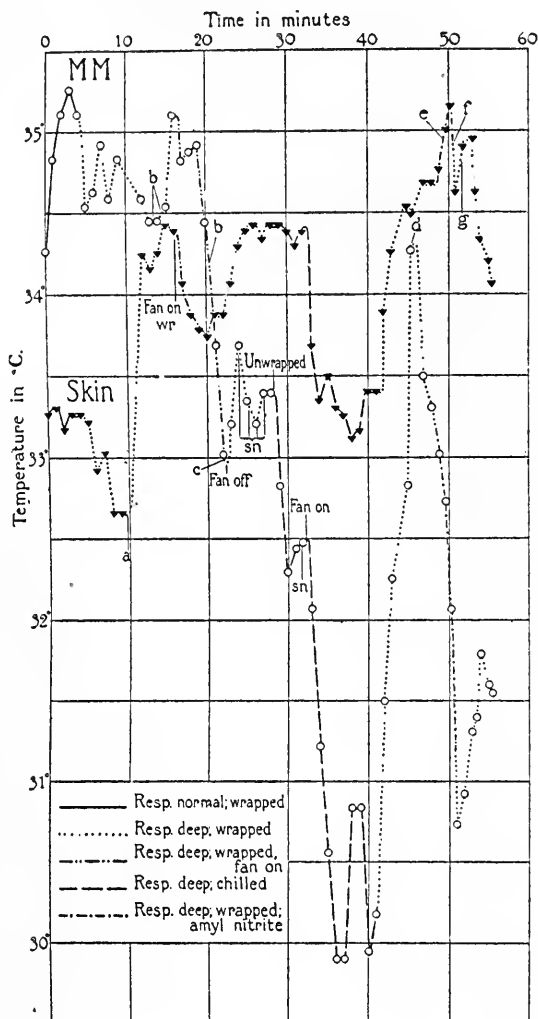
Some difficulty was occasioned in this experiment because the airway of that side of the nasal cavity in which the thermopile was applied became occluded with mucus. Under such conditions mucosa temperature tended to rise without local vasodilation, but snuffing back usually cleared the airway and showed the true course of the temperature curve (Text-fig. 6).

*Experiment 5.*—Subject 1, F. J. C. June 25, 1920, 3 to 4.21 p.m. Application on left nasal septum, 2.8 cm. from opening of nostril. Room temperature  $18\text{--}18.5^{\circ}\text{C}$ .

Text-fig. 7 shows well the effects of graduated chillings. The fan was turned on at 0:16.2 without removing the subject's wraps, which, however, were not quite adequate protection, for the subject felt cold before the fan was turned off. Mucous membrane temperature fell  $2.1^{\circ}\text{C}$ ., an effect in which stray air currents can hardly have been



concerned since the nasal cavity was already being ventilated by every respiration. The skin fall of  $0.65^{\circ}$  was doubtless chiefly due to direct cooling. With the turning off of the fan, skin temperature returned



TEXT-FIG. 7. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of left nasal septum, Experiment 5. *a*, skin thermopile readjusted; *b*, snuffs mucus back twice; *c*, feels cold; snuffs back three times; *sn*, snuffs back; *d*, snuffs back three times; respiration had been too shallow; deepened; *e*, face flushed; *f*, flush gone; stops amyl nitrite; *g*, face very pale; mucous membrane applicator in correct position.

to control level, but mucosa temperature showed only a small recovery. Unwrapping depressed mucosa temperature  $1.1^{\circ}$  without particularly affecting that of the skin. Turning the fan on produced the usual marked fall in skin and mucosa curves, from which there was good

recovery in both instances after rewrapping. The skin rise with amyl nitrite was characteristic; the negative mucous membrane amyl nitrite reaction, due doubtless to hyperpnea and to a fall in general blood pressure, was the only one obtained in the nasal experiments.

Twelve intranasal experiments were completed in the series, with seven different subjects. Without exception there were a clean-cut temperature fall with chilling, and recovery, partial or complete, with rewarming. The sites studied, although all in the anterior half of the nasal chamber, included both the ordinary and the cavernous mucous membrane, and the reactions found—reflex vasoconstriction and ischemia with chilling of the body surface, and vasodilation with rewarming—we believe may be safely regarded as characteristic of at least the respiratory portion of the nasal mucous membrane in general.

The painful irritation of the nasal mucosa incident upon applying the thermopile tips within the nasal cavity caused often also sneezing, lacrimation, and discharge from the nose of clear mucus. The rhinorrhea was more marked usually on the side directly irritated but was present on both sides. It was little if at all affected by the diminished blood supply and shrinkage of the mucous membrane with chilling; of eight experiments in which an attempt was made to estimate whether discharge was more or less during chilling, the result was extremely dubious in four instances; the subject thought that there was more mucus during chilling in three instances and less in one.

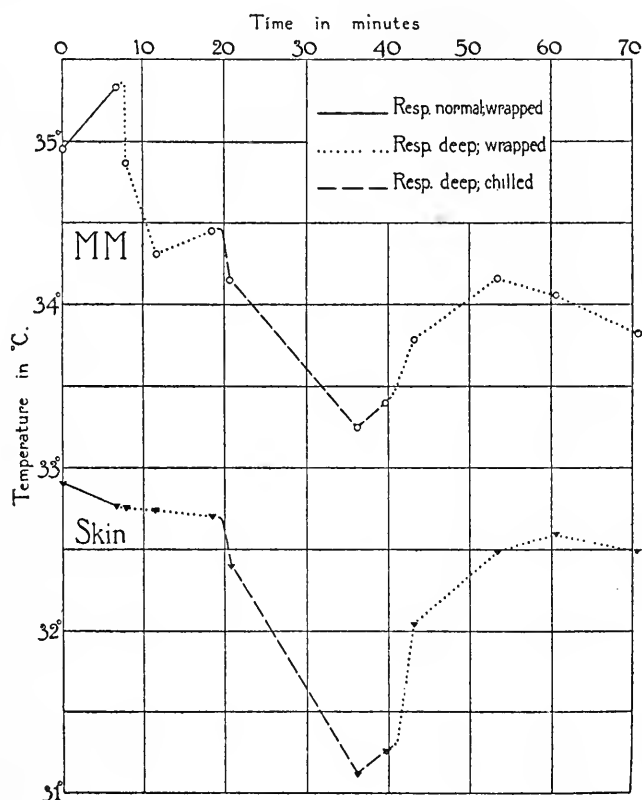
#### *Vasomotor Reactions in the Postnasal Space.*

*Composite Graph.*—The characteristic responses of the nasopharyngeal mucous membrane to chilling and rewarming the body surface are shown in Text-fig. 8, a composite graph of seven experiments. The sites of application were all in the postnasal space, in five instances 3.3 cm., in one 2.8 cm., and in one 3.7 cm. above the posterior margin of the soft palate in the position taken during nose breathing with mouth closed. Four different subjects were used. The points selected for averaging and graphing are the same as those for Text-fig. 3, except that in that part of the nasopharyngeal composite which shows recovery after rewrapping the skin point synchronous with the point of maximum mucous membrane recovery, and the mucous membrane



point synchronous with the point of maximum skin recovery, are also included.

The mucous membrane temperature depression with chilling was  $1.2^{\circ}\text{C}.$ , that of the skin  $1.6^{\circ}\text{C}.$  Recovery, measured from minimal points with chilling to maximal points after rewarming, was  $0.9^{\circ}\text{C}.$  (75 per cent) for mucous membrane,  $1.5^{\circ}\text{C}.$  (94 per cent) for skin. The mucous membrane of the nasopharynx, then, like that of the

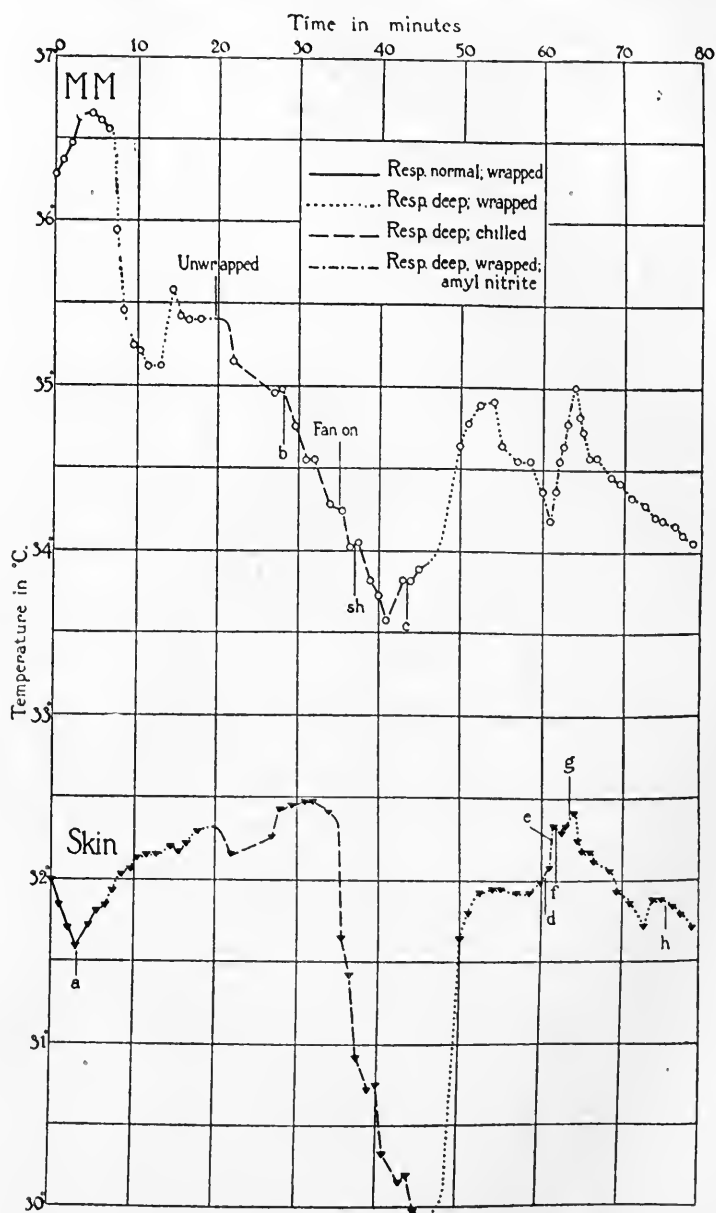


TEXT-FIG. 8. Nasopharyngeal reactions to chilling and rewarming. Temperatures of skin and mucous membrane of postnasal space; composite graphs of Experiments 10, 11, 12, 13, 14, 15, and 16.

oropharynx, and palate, has shown somewhat less tendency than the skin of the forehead to recover its normal blood supply upon rewarming after chilling.

It is to be borne in mind that these observed temperature depressions with chilling are somewhat larger than local vasoconstriction alone would make them; direct cooling contributed to the skin fall as explained above, and a lowering of the temperature of the respired air

by diminished blood supply and shrinkage of the nasal mucous membrane with chilling must have contributed to the temperature fall of

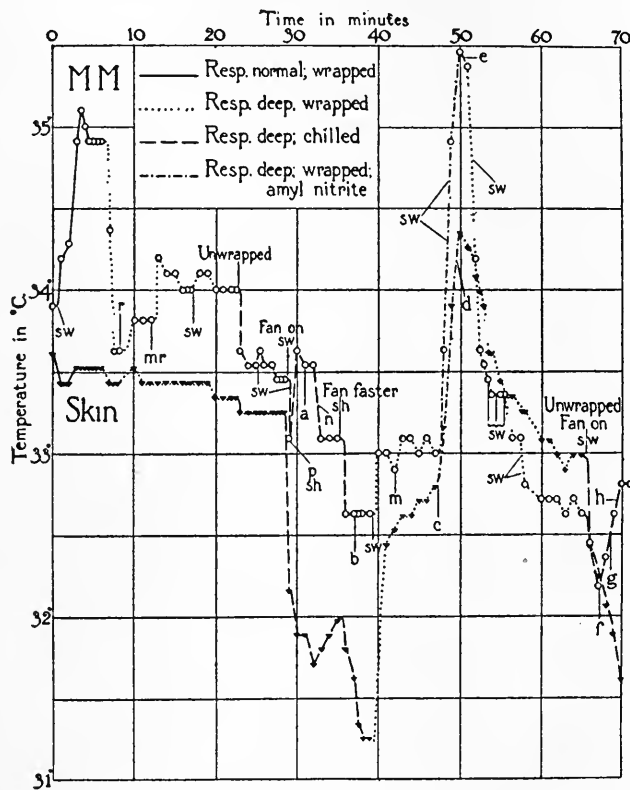


TEXT-FIG. 9. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of postnasal space, Experiment 10. *a*, skin applicator readjusted; *b*, respiration had for several minutes been less deep than control; deepened; *sh*, shivering begins; *c*, has been shivering since started; *d*, starts inhaling amyl nitrite; *e*, face flushing; *f*, stops inhaling momentarily; *g*, throws away amyl nitrite ampule; flushed; *h*, subject feels neither warm nor cool.

the nasopharyngeal mucosa. The latter factor will be further discussed below.

*Experiment 10.*—Subject 5, J. C. McK. July 6, 1920, 2.48 to 4.07 p.m. Application on posterior nasopharyngeal wall, 3.3 cm. above posterior margin of soft palate. Room temperature 19.2–20.3°C.

A low threshold for the chilling vasoconstrictor reflex to the nasopharyngeal mucous membrane, just as with that of the nasal cavity, is shown in Experiment 10 (Text-fig. 9). Unwrapping the subject in the room at a temperature of more than 19°C. was followed by a fall



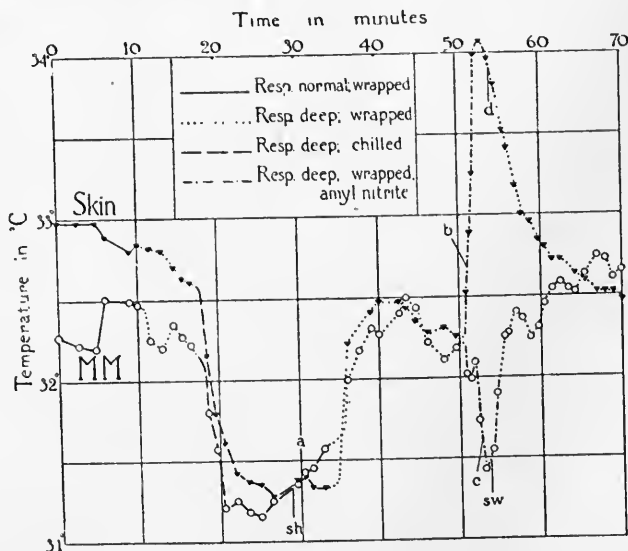
TEXT-FIG. 10. Reactions to graduated chilling, to rewarming, and to amyl nitrite. Temperatures of skin and mucous membrane of postnasal space, Experiment 11. *sw*, swallows; *r*, respiration uncontrolled for about 2 minutes; probably diminished; *mr*, slight movement of pharynx; respiration diminished for about a minute; *p sh*, pain at site of application; begins shivering; *a*, still shivering; fan slows down; *n*, no shivering; *b*, fan still faster; shivering; *m*, muscular contraction of pharynx; *c*, begins amyl nitrite inhalation; swallows five times; *d*, face flushing; *e*, stops inhaling; swallows twice; *f*, swallows three times; pain at site of application; *g*, swallows twice; *h*, swallows; shivering.

of  $1.1^{\circ}\text{C}$ . in the mucous membrane curve, by only a very small ( $0.15^{\circ}$ ) and transitory depression of that of the forehead. Recovery and amyl nitrite reactions are characteristic.

*Experiment 11.*—Subject 6, S. M. July 12, 1920, 10.30 to 11.41 a.m. Application on posterior nasopharyngeal wall, 3.7 cm. above posterior margin of soft palate. Room temperature  $16\text{--}17^{\circ}\text{C}$ .

Skin and mucous membrane temperatures in Text-fig. 10 show step-like depressions corresponding to the gradations in chilling. Interruptions in the mucous membrane fall at 0:29 (*p sh*) and at 0:67.2 (*f*) are synchronous with sensation of pain at the site of application and are doubtless due to movement of the applicator tip against the nasopharyngeal wall, which may cause a slight rise in temperature of the thermopile tips, mechanically, merely by pressing them more firmly against the mucous membrane, and by the irritation causing local vasodilation. The latter is probably much the more important factor. The amyl nitrite rise is very pronounced in each curve,  $2.45^{\circ}\text{C}$ . for mucous membrane and  $1.55^{\circ}\text{C}$ . for skin.

*Experiment 12.*—Subject 7, W. A. H. July 16, 1920, 10.20 to 11.30 a.m. Application on posterior nasopharyngeal wall, 3.3 cm. above posterior margin of soft palate. Room temperature  $18.2\text{--}19^{\circ}\text{C}$ .



TEXT-FIG. 11. Reactions to chilling, rewarming, and amyl nitrite. Temperatures of skin and mucous membrane of postnasal space, Experiment 12. *sh*, begins shivering; *a*, subject's back moistened; *b*, face flushing; *c*, swallows twice; can feel applicator against posterior pharyngeal wall; *sw*, swallows; *d*, flush fading.

The temperature depressions with chilling in Text-fig. 11 are characteristic. The recovery curves are of interest as ascending in each case slightly above control level. One other instance among the eight completed<sup>9</sup> nasopharyngeal experiments showed return of the mucous membrane temperature to control level with rewarming; in the other six experiments recovery was incomplete. The negative amyl nitrite reaction in Experiment 12 is the only one in the nasopharyngeal series.

*Demonstration that Local Decrease of Blood Supply Underlies Observed Depression of Nasopharyngeal Surface Temperature.*—The possibility arose in our minds that the observed depression of temperature of the mucosa surface in the postnasal space consequent upon chilling the body surface might be merely a secondary result of the widening of the airway of the nasal cavity and consequent loss of efficiency in warming the air passing through the nasal cavity into the postnasal space. It is obviously highly improbable *a priori* that the reflex reaction of vasoconstriction and ischemia, the presence of which has been demonstrated in the mucous membranes contiguous above and below the nasopharynx, *i.e.* that of the nose, oropharynx, and palate, should be lacking in the postnasal space. Nevertheless, it was considered worth while to exclude this possibility by definite experimental analysis, and this has been done by the three following sets of observations.

(1) More than doubling the depth of respiration caused less depression of nasopharyngeal mucous membrane temperature than did chilling the body surface. On the other hand, a smaller increase in depth of respiration was followed by a fall in the temperature of the air of the postnasal space more than three times as great as that which followed chilling the body surface. (2) In two parallel sets of experiments, one with application upon the nasopharyngeal and one upon the oropharyngeal mucous membrane, mucosa temperature depression with chilling was somewhat greater in the nasopharynx. The skin

<sup>9</sup> There are in the nasopharyngeal series also one experiment in which temperature depression occurred with chilling but the experiment terminated before rewarming, and three experiments in which the usual reaction to chilling was masked, presumably due to the local vasodilation occasioned by trauma from an ill fitting applicator.

temperature fall, on the other hand, was somewhat greater in the oropharyngeal set, indicating more severe chilling in that set. But it has been demonstrated in former experiments in which the passage of air currents from the nose into the throat was blocked by plugs in the nostrils, and in which the subject breathed through the open mouth,<sup>10</sup> that the oropharyngeal mucous membrane temperature is depressed with chilling because of local diminution of its blood supply. The inference is that the nasopharyngeal temperature fall must also, at least in considerable part, be consequent upon local vasoconstriction and ischemia. (3) The depression of the temperature of the nasopharyngeal mucosa with chilling of the body surface has been greater than the fall in temperature observed under similar conditions in the air of the postnasal space. If the mucosa depression were merely the effect of a cooler air current passing over it from the nasal cavity, the fall of air temperature would have to be considerably greater than that of the mucosa surface cooled by it.

The evidence summarized above may now be given somewhat more in detail.

(1) For each of the seven experiments of the nasopharyngeal composite, measurement was made of the amplitude of excursion of the pneumograph levers on the respiration record. Twenty thoracic and twenty abdominal respirations were measured before, and a like number after the change from normal to deep respiration. The result showed an average increase in respiratory amplitude of 143 per cent; the rate of respiration was kept constant. The mucous membrane temperature depression which followed this increase to more than double respiratory depth amounted to only  $1^{\circ}\text{C}$ ., whereas the mucosa temperature fall with chilling was  $1.2^{\circ}\text{C}$ .

On the contrary, consideration of the two experiments in which the temperature of the air in the postnasal space was followed, shows in one a respiratory increase of 78 per cent, causing a fall in air temperature of  $1.4^{\circ}\text{C}$ . (see Text-fig. 15) and followed by a fall, with chilling, of  $1.3^{\circ}\text{C}$ .; in the other there was an increase in respiration of 191 per cent with corresponding air temperature depression of  $2.95^{\circ}\text{C}$ ., followed by no temperature depression at all with chilling. The average shows an increase of respiratory amplitude of 119 per cent producing a depression in the temperature of the air of the postnasal space of  $2.2^{\circ}\text{C}$ ., and chilling of the body surface producing a depression of only  $0.6^{\circ}\text{C}$ . (see Text-fig. 14).

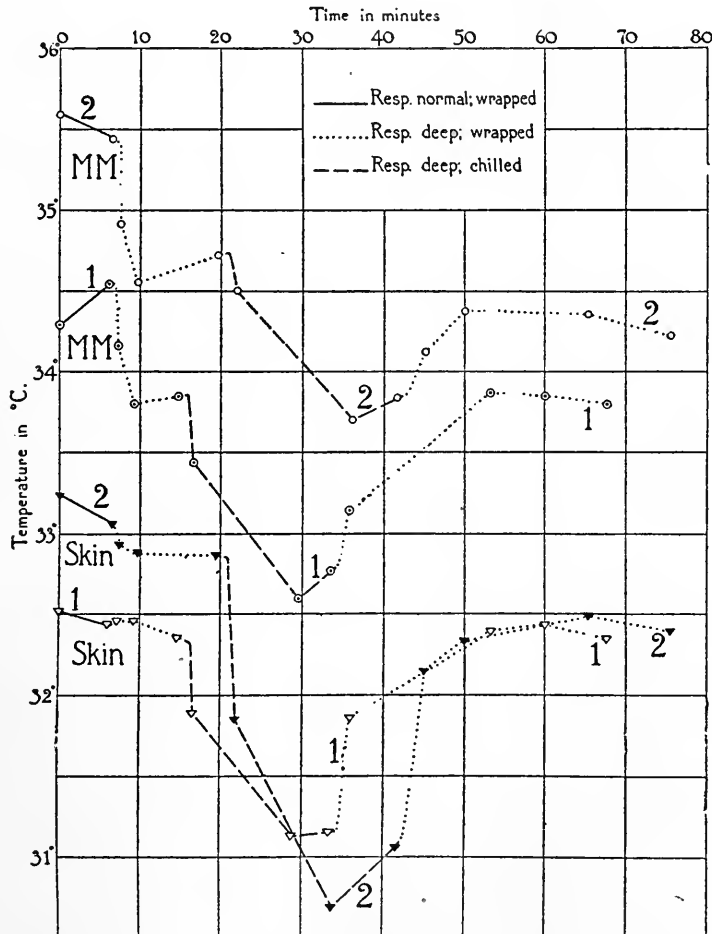
The method used for estimating change in respiratory volume we are aware is not a precise one, but we believe the results sufficiently accurate to justify the

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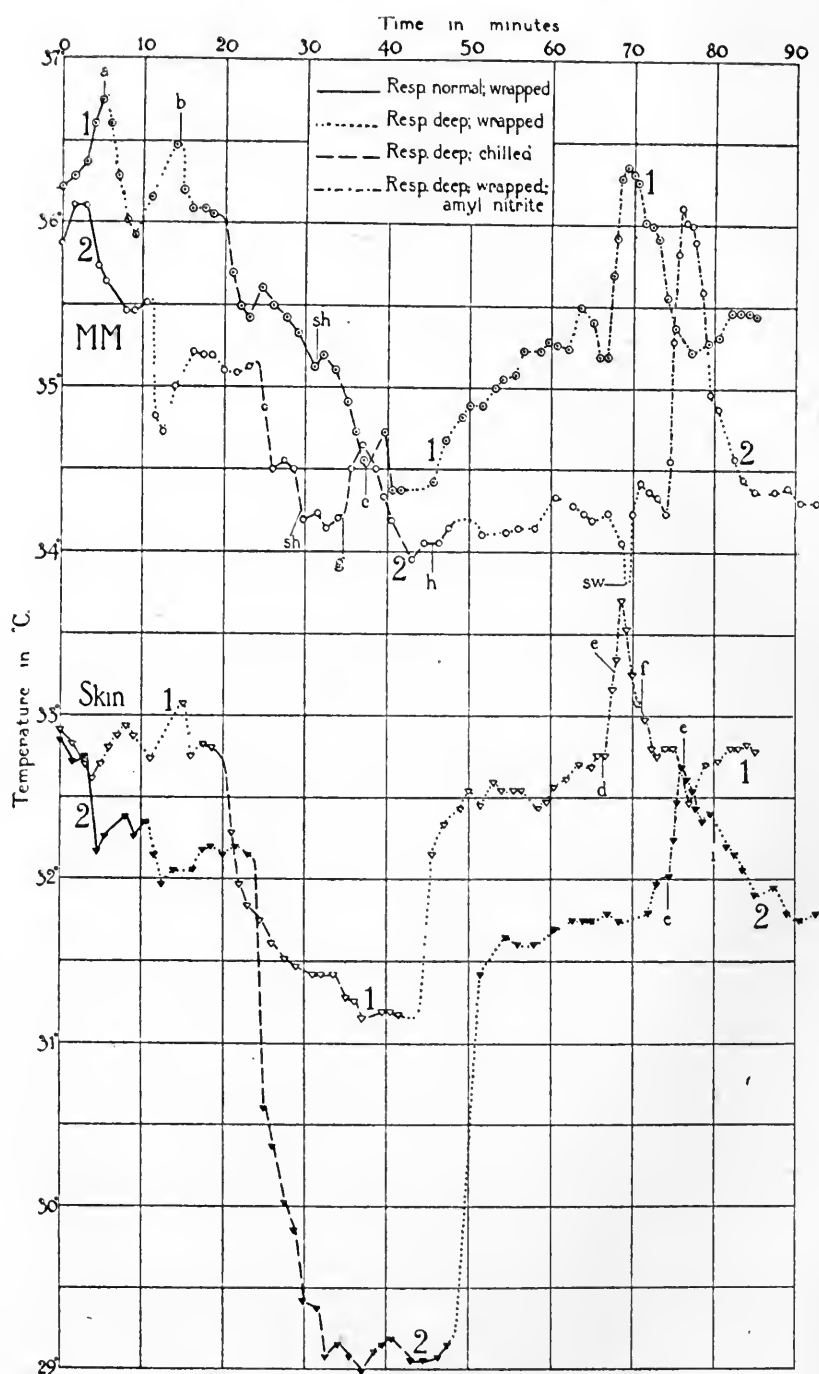
<sup>10</sup> Mudd and Grant,<sup>1</sup> p. 78; Grant, Mudd, and Goldman,<sup>2</sup> p. 92.

conclusion which we draw from them; namely, that the cooling of the air current from the nose into the postnasal space incident upon chilling the body surface is by no means sufficient to explain the observed fall in the surface temperature of the nasopharyngeal mucosa, and that some local change, *i.e.* local diminution of blood supply, is necessarily implied.

(2) The responses of the mucous membranes of the nasopharynx and of the oropharynx, under similar experimental conditions, to chilling and rewarming are shown in Text-fig. 12. Application was made in the nasopharyngeal group 3.3 cm. above the posterior margin of the soft palate of each of three subjects. For the oropharyngeal group application was made upon the posterior pharyngeal wall just below the posterior margin of the soft palate in each of the same three subjects. Experimental conditions were made as much alike as possible in the two sets, except that chilling was a little more severe in the oropharyngeal experiments. The values averaged and plotted in the composite curves are the same as for Text-fig. 8.



TEXT-FIG. 12. Comparison of reactions of nasopharynx and of oropharynx to chilling and rewarming. (1) Temperatures of skin and mucous membrane of postnasal space; composite graphs of Experiments 12, 13, and 14. (2) Temperatures of skin and mucous membrane of oropharynx; composite graphs of Experiments 17, 18, and 19.



TEXT-FIG. 13. Comparison of reactions of nasopharynx and of oropharynx to chilling and rewarming. (1) Temperatures of skin and mucous membrane of postnasal space, Experiment 13. *a*, respiration has been gradually growing shallower; changes to deep respiration; *b*, respiration slightly too shallow; deepened; *sh*, starts shivering; *c*, subject pushes mucous membrane applicator back so that he can feel it touching posterior nasopharyngeal wall; *d*, face flushing; *e*, face flushed; *f*, flush fading. (2) Temperatures of skin and mucous membrane of oropharynx, Experiment 17. *sh*, starts shivering; *g*, cleared throat for first time; still feels applicator touching on original site; *h*, still shivering; has been doing so ever since started; *sw*, swallowed for first time; with swallow, mucous membrane temperature rose 0.5°C.; *e*, face flushed; *i*, flush gone.



Skin depression with chilling was  $1.2^{\circ}\text{C}.$  in the nasopharyngeal set, as compared with  $2.2^{\circ}\text{C}.$  in the oropharyngeal experiments, a consequence, presumably, of the more severe chilling in the latter. Yet the corresponding depression of temperature of the nasopharyngeal mucosa was  $1.25^{\circ}\text{C}.$ , that of the oropharynx, only  $1^{\circ}\text{C}.$  But the oropharynx, as mentioned above, had already been shown to possess the power of reacting with vasoconstriction and ischemia to chilling. If then the oropharynx under the influence both of a cooler air current and of local diminution of blood supply falls in temperature only  $1^{\circ}$ , it can hardly be supposed that the nasopharynx would fall  $1.25^{\circ}$  under the influence of a cooler air current alone, and the implication is again that the nasopharynx also must suffer a decrease in blood supply.

Two of the individual experiments of which the composite is made up are shown in Text-fig. 13.

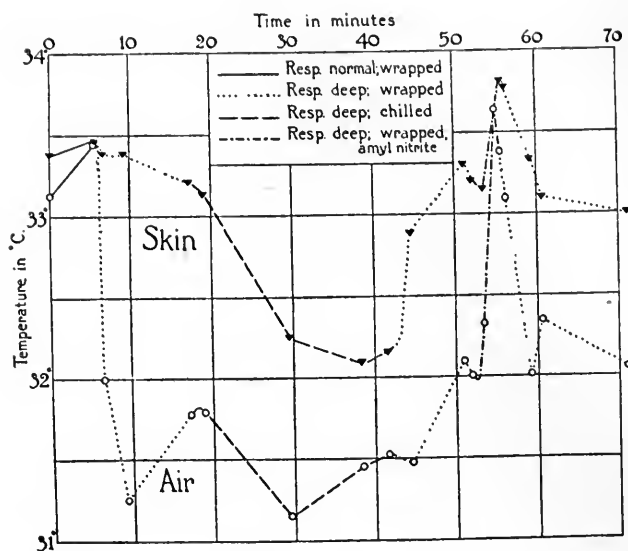
*Experiment 13.*—Subject 8, G. D. S. July 6, 1920, 11.17 a.m. to 12.42 p.m. Application on posterior nasopharyngeal wall, 3.3 cm. above posterior margin of soft palate. Room temperature  $18.5\text{--}19^{\circ}\text{C}.$

*Experiment 17.*—Subject 8, G. D. S. July 10, 1920, 9.05 to 10.38 a.m. Application on posterior oropharyngeal wall, just below posterior margin of soft palate. Room temperature  $18.2\text{--}19^{\circ}\text{C}.$

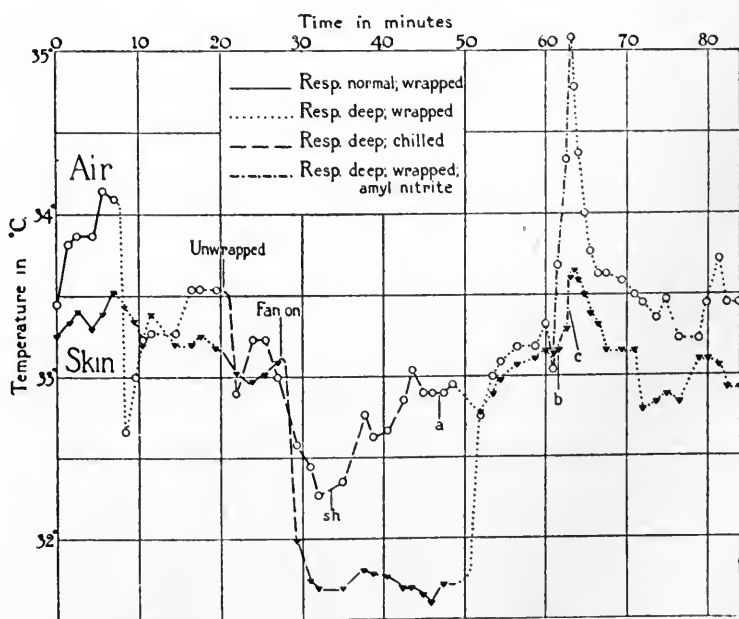
Experiments 13 and 17 are characteristic in their temperature depressions with chilling, partial recoveries with rewarming, and sharp rises with amyl nitrite. The skin depression in the nasopharyngeal experiment is  $1.65^{\circ}\text{C}.$  and that in the oropharyngeal experiment  $3.2^{\circ}\text{C}.$ , as contrasted with corresponding mucous membrane temperature falls of  $1.7^{\circ}$  in the nasopharynx and  $1.2^{\circ}$  in the oropharynx.

(3) Variations in the average temperature of the air of the postnasal space are shown in Text-fig. 14. The curves are a composite of two experiments under conditions as closely similar as we could make them to those of the series graphed in Text-fig. 12, and with two of the same subjects. The values averaged and plotted are as usual the first and last readings of the experiment, the points immediately before and after each change of conditions, and the points of maximum response to changed conditions. In this graph, however, the skin points synchronous with mucous membrane minimum and maxima and the mucous membrane points synchronous with skin minimum and maxima are included. The response to amyl nitrite is also shown.

The thermopile tips were held in the air of the postnasal space by binding them upon the anterior side of the vertical arm of the nasopharyngeal applicator with the vertical arm 3.3 cm. in length, bending this arm slightly forward, and preventing its touching the posterior nasopharyngeal wall by a small pad of adhesive plaster on its posterior surface. With each respiration the galvanometer reading usually swung through from 4 to 8 mm., falling with inspiration and rising with expiration, and the observer tried to take the mean value as his reading in each case. The difference thus indicated ( $0.36\text{--}0.72^{\circ}\text{C}.$ ) between the temperatures of inspired and expired air is, because of the lag of the apparatus, probably considerably less than the actual difference.



TEXT-FIG. 14. Effects of deepened respiration, chilling, rewarming, and amyl nitrite. Temperatures of skin and air of postnasal space; composite graphs of Experiments 20 and 21.



TEXT-FIG. 15. Effects of deepened respiration, chilling, rewarming, and amyl nitrite. Temperatures of skin and air of postnasal space, Experiment 20. *sh*, starts shivering; *a*, still shivering; *b*, face flushing; *c*, face flushed.

The skin temperature depression with chilling shown in Text-fig. 14 is  $1.1^{\circ}\text{C.}$ , almost equal to the fall of  $1.2^{\circ}\text{C.}$  in the comparable series of Text-fig. 12. The maximum depression of the temperature of the air of the postnasal space, however, is only  $0.6^{\circ}\text{C.}$  as contrasted with a fall of  $1.25^{\circ}$  in the temperature of the mucosa surface in the postnasal space. Mucosa cooling once more, then, cannot be explained merely by air cooling, which to be a sufficient cause would have to be several times as great.

*Experiment 20.*—Subject 8, G. D. S. July 20, 1920, 1.18 to 2.42 p.m. Thermopile tips in air of postnasal space, 3.3 cm. above posterior margin of soft palate. Room temperature about  $20^{\circ}\text{C.}$

Text-fig. 15 shows one of the two individual experiments from which the composite graph of Text-fig. 14 is made. The fall in air temperature shown here is  $1.3^{\circ}\text{C.}$ , twice that of the composite. The composite value is almost undoubtedly too small because of the averaging in of Experiment 21 in which no air temperature depression with chilling was observed. The failure of the air current to cool at all in Experiment 21 we do not know how to explain unless there was a very slight decrease in respiratory amplitude synchronous with chilling, an hypothesis which study of the respiration record leaves uncertain, since the excursion of the thoracic lever became slightly less and that of the abdominal lever slightly greater when chilling began. Upon rewarming in Experiment 21 air temperature rose  $0.8^{\circ}\text{C.}$  in 4 minutes, thus agreeing with the theoretical expectation.

Text-fig. 15 probably represents more nearly than the composite of Text-fig. 14 the true picture of changes in the temperature of the air of the postnasal space due to deepened respiration, chilling, rewarming, and amyl nitrite.<sup>11</sup>

#### *Local Differences in the Vasoconstrictor Reflex with Slight Cutaneous Chilling.*

The chilling caused by completely disrobing the very warmly wrapped subject in a room the temperature of which was slightly below ordinary room temperature seems to have been close to the minimal

<sup>11</sup> An interesting instance, presumably due to decreased blood supply and shrinkage of the nasopharyngeal mucous membrane in response to cutaneous chilling, occurred in July, 1920. The father of one of us, Dr. H. G. M., had been troubled with chronic inflammation of the nasopharyngeal mucous membrane, and in June, 1920, had the mucous membrane cauterized. The swollen membrane at the site of cauterization for some time thereafter on occasion occluded the opening of the left Eustachian tube. Coming in from the golf links one very hot day, he had the congested feeling in his left ear indicative of Eustachian occlusion. Upon getting into a cold shower bath, the feeling of congestion promptly disappeared.

TABLE I.  
*Comparison of Vasoconstrictor Reflexes to Various Peripheral Areas with Slight Cutaneous Chilling.*

Experi- ment No.	Year.	Subject.	Room temperature.	Skin site.	Maximum skin tempera- ture fall.	Mucous membrane site.	Maximum mu- cous membrane temperature fall.	Remarks.
			°C.		°C.		°C.	
2	1918	S. M.	20.3-20.75	Left infraclavic- ular fossa.	2.2	Hard palate.	1.5	Respiration not con- trolled.
3	1918	S. B. G.	18.25-18.9	Left supracla- vicular fossa.	2.5	Soft	1.4	Respiration not con- trolled.
6	1918	G. A.	19.05-19.7	Left supracla- vicular fossa.	1.4	"	0.05	Respiration partially con- trolled.
5	1918	S. B. G.	19.1-19.55	Left supracla- vicular fossa.	1.95	"	0.6	Respiration not con- trolled.
7	1918	S. M.	17.6-18.8			Oropharynx.	0.9	Respiration partially con- trolled.
4	1918	S. M.	19.3-20.6	Left supracla- vicular fossa.	0.7	"	-1.2*	Respiration not con- trolled.
22	1920	S. M.	18-19	Forehead.	0.1	"	0.4	Respiration controlled.
23	1920	S. B. G.	17 (approx- imate).	"	-0.3	Right tonsil.	1.2	"
11	1920	S. M.	16-17	"	0.1	Nasopharynx.	0.55	"
10	1920	J. C. McK.	19.2-20.3	"	0.15	"	1.1	"
4	1920	S. B. G.	14 (approx- imate).	"	0.2	Left inferior turbinate.	2.0	"
2	1920	A. G.	16-17	"	0.25	"	2.2	"
7	1920	S. B. G.	16.5 (ap- prox- imate).	"	0.2	" nasal septum.	2.1	"

9	1920	A. G.	16.5-16.8	Forehead.	0.4	Left middle meatus.	1.25	Respiration controlled.
3	1920	M. F. W.	16.5-18	"	-0.02	"	1.5	"
8	1920	S. M.	17 (approximate).	"	0.2	Right inferior turbinate.	0.3	"
24	1920	W. R. M.	17-18	"	0.25	" nasal septum.	5.0	"
6	1920	S. M.	17-18	"	0.2	"	(approximate).	"
5	1920	F. J. C.	18-18.5	"	0.1	"	0.6	"
21	1920	S. M.	18.5-18.8	"	-0.2	Left	1.1	"
20	1920	G. D. S.	19.8 (approximate).	"	0.2	Air of postnasal space.	-0.65	"
						"	0.6	"

Average maximal temperature depressions caused by reflex vasoconstriction in response to chilling of body surface by disrobing warmly wrapped subject: skin at base of neck, 1.74°C.; skin of forehead, 0.13°C.; mucous membrane of palate and oropharynx (omitting Experiment 4, 1918), 0.82°C.; mucous membrane of nasopharynx, 0.83°C.; mucous membrane of nasal cavity, 1.78°C.; mucous membrane of palatine tonsil (one experiment), 1.2°C.

\* In those experiments in which the temperature rose instead of falling with unwrapping, the point of maximum rise is preceded by a minus sign.

value of stimulation requisite to bring about reflex peripheral vasoconstriction, and it has been possible by this means to demonstrate a difference between the threshold of the vasoconstrictor reflex to the skin of the forehead on the one hand, and to the skin at the base of the neck and to the mucous membranes of the nose, throat, and palate on the other. Such mild chilling caused vasoconstriction and depression of superficial temperature in the skin of the supra- and infraclavicular fossæ and in the mucous membranes of the palate, tonsil, oropharynx, nasopharynx, and nasal cavity. In fifteen experiments in which its effect on the skin of the forehead has been observed, on the other hand, a slight rise in temperature in three instances, rather than a fall, was observed in the interval during which the subject was unwrapped but not exposed to the draft of the fan; of the other twelve experiments, in about half the relation between temperature fall and unwrapping was uncertain, and the slight depression observed may have been due to accidental variation; in the remainder the temperature fall seemed clearly to be referable to the slight chilling, but was very small,  $0.1-0.25^{\circ}\text{C}$ . Under the stronger stimulation of chilling with the fan the forehead showed clear-cut vasoconstriction.

It is quite probable that the figures given in Table I, for mucous membrane temperature fall in the palatine-oropharyngeal group, are somewhat larger than they should be; these experiments were performed before we realized that the involuntary deepening of respiration with chilling necessitated careful respiratory control.<sup>12</sup>

A single experiment,<sup>13</sup> not included in Table I but the results of which seem sufficiently clear-cut to warrant acceptance, was performed in 1918 with one thermopile on the normal skin of the thorax and the second upon the scar at the site of a breast amputation performed about  $3\frac{1}{2}$  months previously. The subject was chilled merely by unwrapping from the waist up at a room temperature of  $15.65-16.35^{\circ}\text{C}$ . The scar temperature fell  $1.8^{\circ}\text{C}$ ., the skin temperature  $2.6^{\circ}\text{C}$ . in 5 minutes.

From the above data it seems safe to conclude that the threshold of the vasoconstrictor reflex in response to chilling of a distant cutaneous

<sup>12</sup> Mudd and Grant,<sup>1</sup> p. 69.

<sup>13</sup> Mudd and Grant,<sup>1</sup> Experiment 25, p. 94, and Fig. 14, middle curves.

area is higher for the skin of the forehead than for the skin of the covered portions of the trunk and the mucous membranes of the nasal cavity, postnasal space, throat, and palate.

*Local Differences in the Recovery of Blood Supply upon Rewarming after Chilling.*

Skin and mucous membrane blood supply is diminished in the sites studied as a quick reflex response to sudden cutaneous chilling, and is increased in prompt response to rewarming. But the recovery of blood supply has not typically been complete in any of the mucous membrane sites studied except the palatine tonsils. Two questions remain therefore to be answered: (1) To what degree is blood supply fairly promptly restored in the several sites under consideration? (2) What is the duration after cessation of chilling of the ischemia of the mucous membranes?

It is perhaps worth enumerating instances of the difficulties which beset an attempt to answer these questions and make us offer conclusions with extreme diffidence. We can never be perfectly certain that the conditions of application of the thermopile tips are identical before chilling and during rewarming a half hour or more later. Although the respiration charts are kept and studied, it is impossible to be sure that the respiratory ventilation of the mucous membranes remains constant within sufficiently narrow limits to make temperature differences of one- or two-tenths of a degree, separated by a long interval of time, significant. It was impossible to rewrap the subject precisely as he had been wrapped before the experiment. Superficial temperature and vasomotor tone do not run precisely parallel over such long intervals of time as are here under consideration. The variability in degree of recovery among the experiments is such that much more data than are available would have to be accumulated in order to form a basis for precise quantitative conclusions. Finally, we cannot be sure to just what degree such fine details of reaction as are now under consideration, duplicate, under experimental conditions, reactions as they would occur under natural conditions.

However, it is possible, with the above reservations always in mind, from analysis of the available data to derive a mathematical expression which expresses in some degree satisfactorily the relative tendencies of skin and mucous membranes to recover their blood supplies upon rewarming after cutaneous chilling, and this is done in Table II.

TABLE II.

*Recovery of Blood Supply at Various Sites upon Rewarming after Chilling of the Body Surface.*

Experiment No.	Year.	Subject.	Mucous membrane site.	Maximum mu- cous membrane temperature fall.	Maximum mu- cous membrane recovery.	Time required for maximum recovery.	Maximum skin temperature fall.	Maximum skin recovery.	Time required for maximum recovery.	Duration of ex- periment after wrapping.
				°C.	°C.	min.	°C.	°C.	min.	min.
15	1918	S. B. G.	Soft palate.	1.5	0.8	11	0.25	0.45	12.5	15.5
18	1918	A. G.	"	1.9	0.65	15.5	2.35	1.75	9.5	17
19	1918	S. M.	" "	0.8	0.2	6.5	2.0	3.0	9.5	14.5
12	1919	A. G.	" "	0.8	0.3	8	2.4	2.1	23	25
13	1919	S. B. G.	" "	0.75	0.6	12	2.1	2.3	5	19
Average . . . . .				1.15	0.5	10.6	1.8	1.9	11.9	18.2
Recovery index = 0.42										
16	1918	S. M.	Oropharynx.	1.5	1.3	18	3.3	3.9	14	18
17	1920	G. D. S.	"	1.2	0.5	22.5	3.2	3.0	24.5	44
19	1920	W. A. H.	"	1.4	0.9	38.5	1.5	1.25	42	47.5
Average . . . . .				1.3	0.9	26.3	2.7	2.7	26.8	36.5
Recovery index = 0.64										
21	1918	S. M.	Nasopharynx.	1.8	1.45	6	1.95	1.85	4	13
10	1920	J. C. McK.	"	1.8	1.3	7.5	2.3	2.0	13.5	32.5
11	1920	S. M.	"	1.4	0.5	3.5	2.1	1.7	24.5	25.5
12	1920	W. A. H.	"	1.1	1.6	33	1.3	1.2	6	36
13	1920	G. D. S.	"	1.7	1.1	21	1.65	1.55	21	42.5
14	1920	S. M.	"	1.0	1.1	3.5	0.7	1.0	3.5	22.5
15	1920	S. M.	"	0.7	0.4	3	1.0	0.8	8	10
16	1920	W. A. H.	"	0.7	0.45	16	2.0	1.8	20.5	21.5
Average . . . . .				1.3	1.0	11.6	1.6	1.5	12.6	25.4
Recovery index = 0.84										
20	1918	S. M.	Left tonsil.	1.2	(3.9)*	7.5	2.0	1.8	5.5	20
				1.3	1.5	13	0.9	1.45	8	13
1	1919	W. G. E.	" "	1.9	3.6	36.5	3.1	3.1	38	42.5
3	1919	S. B. G.	" "	1.0	0.95	7	2.4	2.3	7	35.5
5	1919	S. B. G.	" "	1.7	1.5	3	4.1	3.6	5	6
23	1920	S. B. G.	Right "	1.5	1.0	7.5	2.0	1.4	9.5	11.5
30	1920	A. L. E.	" "	0.3	2.35	5.5	2.9	3.3	23	
31	1920	S. B. G.	" "	1.2	1.2	3.5	1.1	0.8	14.5	15.5
Average . . . . .				1.25	2.0	10.4	2.3	2.2	13.8	20.6
Recovery index = 1.66										

\* Interpreted in 1918 as an experimental error; now regarded as probably representing a true reaction of hyperemia.



TABLE II—*Concluded.*

Experiment No.	Year.	Subject.	Mucous membrane site.	Maximum mu- cous membrane temperature fall.	Maximum mu- cous membrane recovery.	Time required for maximum recovery.	Maximum skin temperature fall.	Maximum skin recovery.	Time required for maximum recovery.	Duration of ex- periment after wrapping.
				°C.	°C.	min.	°C.	°C.	min.	min.
2	1920	A. G.	Left inferior tur- binate.	6.3	6.6	37.5	3.9	3.5	24.5	49.5
3	1920	M. F. W.	Left middle mea- tus.	3.3	4.5	12.5	1.3	1.1	10	30
4	1920	S. B. G.	Left inferior tur- binate.	6.5	4.65	9	2.3	2.2	10	24.5
6	1920	S. M.	Right nasal sep- tum.	3.65	1.2	2.5	2.9	2.4	28.5	36.5
7	1920	S. B. G.	Left nasal septum.	4.15	3.5	27	1.3	0.5	2.5	40.5
8	1920	S. M.	Right inferior tur- binate.	6.6	3.9	8.5	3.0	1.7	7.5	28
9	1920	A. G.	Left middle mea- tus.	5.5	1.45	11.5	1.8	1.45	8	30
25	1920	S. M.	Right inferior mea- tus.	1.0	0.4	3	2.3	1.4	10	14
28	1920	A. G.	Right middle tur- binate.	4.0	2.8	8	1.5	1.4	2	9
Average .....				4.6	3.2	13.3	2.25	1.7	11.4	29.1
Recovery index = 0.92										

In all the experiments in Table II respiration was controlled and the site of skin application was the forehead. From the data tabulated the percentages of skin and mucous membrane recovery are readily computed, and from these the recovery index according to the following formula:

Mucous membrane recovery

$$\frac{\frac{\text{Mucous membrane fall}}{\text{Skin recovery}}}{\text{Skin fall}} = \frac{\text{percentage mucous membrane recovery}}{\text{percentage skin recovery}} = \text{recovery index}$$

The "recovery index" is thus an expression of the tendency of the mucous membrane to recovery of its blood supply referred to that of the skin taken as unity, and at least eliminates in considerable degree

effects due to chance differences in the rewapping of the subjects, and to alterations in general blood temperature and pressure.

Even though we bear in mind, then, all the reservations above, we believe that study of Table II necessitates again the conclusions which we reached from these and other data in 1918<sup>14</sup> and again in 1919;<sup>15</sup> namely, that palate and oropharynx have in our experiments exhibited considerably less tendency than the skin to regain their blood supply on rewarming after chilling, and remain for some time at least somewhat ischemic; and that the palatine tonsils have exhibited greater tendency than the skin to recover blood supply after cessation of chilling, and in several instances have become actually hyperemic.<sup>16</sup> The nasopharynx and nasal cavity, although the available data are hardly sufficient to be conclusive on this point, seem to occupy an intermediate position, and have, on the average, exhibited only a little less tendency than the skin to recover blood supply. The skin has sometimes regained a little more than its control blood supply, sometimes a little less; on the average it has returned about to control conditions; its temperature recovery for the 33 experiments tabulated has been 92 per cent.

Only a few experiments have been sufficiently prolonged after rewapping to throw much light on how long the mucous membranes may remain ischemic. The following experiments (Table II) are probably most significant in this respect.

Experiment 13 (1920), nasopharynx (Text-fig. 13); maximum normal temperature recoveries of 67 per cent for mucous membrane and of 94 per cent for skin, reached in 21 minutes from the time of rewapping. Temperatures were elevated by amyl nitrite, but thereafter returned to about the levels indicated. Duration of experiment after cessation of chilling, 42.5 minutes. Recovery index = 0.71.

Experiment 17 (1920), oropharynx (Text-fig. 13); maximum normal temperature recovery of 40 per cent for mucous membrane, reached in 22.5 minutes, and of 94 per cent for skin in 24.5 minutes. Rise with amyl nitrite; return thereafter to about levels indicated. Duration after cessation of chilling, 44 minutes. Recovery index = 0.43.

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<sup>14</sup> Mudd and Grant<sup>1</sup>, p. 74.

<sup>15</sup> Grant, Mudd, and Goldman,<sup>2</sup> p. 93.

<sup>16</sup> This reaction of tonsillar hyperemia with warming has also been found by Azzi (Azzi, A., *Riforma med.*, 1921, xxxvii, 175) in experiments in which he repeated, confirmed, and extended our studies upon the tonsils.

Experiment 19 (1920), oropharynx; maximum temperature recovery of 66 per cent in 38.5 minutes for mucous membrane, and of 83 per cent in 42 minutes for skin. Duration after cessation of chilling, 47.5 minutes. Recovery index = 0.79.

It seems probable that a slight loss of body heat after cessation of chilling<sup>17</sup> may have accounted in part for the failure of the temperatures to rise higher, but this should affect skin and mucous membranes practically equally and could not account for the low recovery index of the mucous membranes.

#### DISCUSSION.

Tschalusow<sup>18</sup> inserted a tube, wrapped with vaseline-soaked cotton and connecting with a tambour, into his anterior nares, and packed his posterior nares. With his nose thus acting as a plethysmographic chamber, he studied the effect of various stimuli, as follows: (1) insertion of the legs half-way up the shin in water of 18°C.; (2) immersion in water of 40–41°C.; (3) electric stimulation; (4) needle pricks; (5) scratching of the skin of the lower extremity. All the stimuli resulted in essentially one effect—contraction of the nasal vessels. The most clear-cut and constant effects were given by the cold water.

Schade<sup>19</sup> has tabulated the reflex effects of chilling and the reactions to adrenalin administration upon the various organs within the motor distribution of the true sympathetic nervous system and finds the two, qualitatively at least, practically identical. Although we have made no attempt to study adequately the reaction of the mucous membranes to adrenalin, the one instance which was observed and recorded in 1918<sup>20</sup> showed a transient fall of 2°C. in the temperature of the palatine mucous membrane of a dog with adrenalin, and the rapid loss of body heat in the anesthetized animals was temporarily checked by adrenalin injection, doubtless due in part to cutaneous vasoconstriction. Further study would probably confirm this reaction of mucous membrane ischemia with adrenalin, since the vasoconstrictor fibers for the head are contained in the cervical sympathetic nerve.<sup>21</sup> The reactions to cutaneous chilling we have demonstrated thus constitute one more illustration of the reflex stimulation of the motor elements of the sympathetic system through chilling of the body surface.

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<sup>17</sup> Mudd and Grant,<sup>1</sup> p. 66.

<sup>18</sup> Tschalusow, M. A., *Arch. ges. Physiol.*, 1913, cli, 540.

<sup>19</sup> Schade, H., *Z. ges. exp. Med.*, 1918–19, vii, 355; *Münch. med. Woch.*, 1919, lxvi, 1021.

<sup>20</sup> Mudd and Grant<sup>1</sup>, p. 60.

<sup>21</sup> Schäfer, E. A., *Text-book of physiology*, Edinburgh, 1900, ii, 141. Nagel, W., *Handbuch der Physiologie des Menschen*, Braunschweig, 1909, iv, 405.

The relation of this work to the excitation of upper respiratory infection has been fully discussed elsewhere<sup>22</sup> and we will not consider it here beyond reaffirming our belief in the probability of the hypothesis that the mucous membrane ischemia incident on prolonged or excessive chilling, especially if combined with overfatigue or loss of sleep or other cause of lowered resistance, may mediate infection by the indigenous pathogenic microorganisms.<sup>23</sup>

#### SUMMARY.

Devices are described by means of which the terminals of thermopiles may be held in stable apposition with the mucous membranes of the nasal cavity and postnasal space and local temperature variations thus followed.

Chilling of the body surface has without exception caused depression of the temperature of the nasal mucosa surface, amounting in some instances to as much as 6°C., and indicating marked reflex vasoconstriction and diminution of blood supply. With rewarming, partial recovery of blood supply promptly occurs, although recovery has been incomplete within the duration of the experiments in ten of twelve instances.

Application of the wires within the nasal cavity has usually caused pain and discharge of clear mucus, sometimes also lacrimation and sneezing. The rhinorrhea has occurred both on the side directly irritated and on the opposite side, although on the former more abundantly, and has apparently been little if at all affected by the diminished blood supply and shrinkage of the mucous membrane incident to chilling the body surface. Discharge from the nose has been at most a rare occurrence in experiments in which the nasal mucosa was not directly irritated.

The temperature of the nasopharyngeal mucosa surface has also been depressed, typically between 1° and 2°C., with chilling of the

<sup>22</sup> Mudd, S., Grant, S. B., and Goldman, A., *J. Lab. and Clin. Med.*, 1921, vi, 175, 253, 322; *Ann. Otol., Rhinol. and Laryngol.*, 1921, xxx, March number.

<sup>23</sup> The chilling in our studies has always been sudden. Under circumstances in which it is very gradual, as for instance when a person sleeps in a bedroom the temperature of which falls slowly through the night, as Dr. F. C. Shattuck has suggested to one of us, we should expect less vasomotor reaction and less disturbance of the normal distribution of the blood.

body surface. This depression has also been shown to have local reflex vasoconstriction and ischemia as its basis. With rewrapping, prompt return toward normal occurs, but here also recovery of blood supply has in the majority of instances not been complete within the duration of the experiments.

The thresholds of the chilling vasoconstrictor reflex to the mucous membranes of the nasal cavity and postnasal space, tonsil, oropharynx, and palate and the threshold of the reflex to the skin of the trunk have been found to be lower than the threshold of the like reflex to the skin of the forehead. Disrobing the warmly wrapped subject in a room a little below ordinary room temperature has been found sufficient to cause marked vasoconstriction in the sites of the former group, but only slight or no vasoconstriction in the forehead.

A number of instances of cold or sore throat occurred among the subjects of the experiments, in several instances correlated with somewhat interesting bacteriologic findings which will be described elsewhere.<sup>24</sup>

It is a pleasure to thank the friends whose aid as subjects has made the present study possible.

<sup>24</sup> Goldman, A., Mudd, S., and Grant, S. B., *J. Infect. Dis.*, 1921, xxix (in press).



## THE CONCENTRATING ACTIVITY OF THE GALL BLADDER.

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The experiments here to be reported were undertaken as the result of observations upon stasis bile collected after ligation of the common duct. The accumulation of pigment in such bile seemed to us to indicate that some part of the duct system possesses a concentrating faculty of considerable moment for pathological processes.

It is current knowledge that bladder bile is normally thicker than the secretion as it comes from the liver. The point finds a brief mention in text-books. But the significance accorded it may be judged from the fact that for quantitative studies of the bile from day to day the gall bladder is regularly utilized as a link in the fistulous channel connecting the hepatic duct with the body surface.

Maly<sup>1</sup> states that liver bile from the dog has 3.5 to 4.9 per cent of dry substance, and that from the bladder over 20 per cent. The latter fluid, according to Hoppe-Seyler's analyses which are quoted by Maly *in extenso*, yields far the greater quantity of bile salts. Brand<sup>2</sup> found 1 to 4 per cent of solids in the fistula bile of human beings, and as much as 20 per cent in the bladder contents. According to Hammarsten<sup>3</sup> a part of the water of the bile is abstracted and a mucinous nucleoprotein added by the bladder. It is probable that this organ empties itself only partially upon contraction, and the secretion may remain in it as in a sort of backwater to be acted upon over long periods of time. Because of uncertainty as regards these matters, comparative analyses such as those just given bear but obliquely on the problem of the rapidity of the changes undergone by the bile.

In the present paper we shall deal solely with the influence of the gall bladder upon the bile, reserving for an accompanying one the influence of the ducts.<sup>4</sup>

<sup>1</sup> Maly, R., in Hermann, L., *Handbuch der Physiologie*, Leipsic, 1881, v, pt. 2, 172.

<sup>2</sup> Brand, J., *Arch. ges. Physiol.*, 1902, xc, 491.

<sup>3</sup> Hammarsten, O., *Lehrbuch der physiologischen Chemie*, Wiesbaden, 8th edition, 1914, 411.

<sup>4</sup> Rous, P., and McMaster, F. D., *J. Exp. Med.*, 1921, xxxiv, 75.

*Method.*

The best method of study will be one whereby a bile of known constitution is supplied through the normal channels to an intact gall bladder by the animal's own liver. It is practicable in the dog owing to the arrangement of the ducts. By means of a single ligature appropriately placed, a type sample of bile can be diverted for separate collection, while the remainder flows to the bladder.

The common duct of the dog is formed as a rule by the union of three large channels, and high up into the middle one the gall bladder empties. That on the right hand is derived from the caudate and right lateral and central lobes. By its entrance a few centimeters above the duodenum the common duct is finally formed, the other channels uniting much nearer the liver. If their derivative duct is tied just above where this final duct enters, all of the bile from the major portion of the hepatic tissue is pent up and directed into the gall bladder, whereas the secretion from the caudate and right lateral lobes still reaches the common duct and may be collected through a cannula. Here, in essence, is the plan of our experiments.

Owing to duct anomalies, the partition of bile effected by a ligature placed as described may vary considerably. We have made careful dissections at autopsy, tracing out each duct and ultimately determining by weight the amount of tissue delivering bile to either side of the ligature. The liver of the dog is so deeply cleft that usually this can be done accurately. But when a single lobe has ducts running to both sides of the ligature, as not infrequently happens, the partition of the tissue can be only approximately learnt.

Vigorous dogs with a wide costal angle were chosen. Under ether, a sufficient dissection was made to tie a small glass cannula into the common duct and to place higher up the essential, or partitioning, ligature, as we shall henceforth term it. In our first attempt a considerable segment of duct was freed and the gastrohepatic omentum subjected to trauma, with result that almost no bile was secreted in the 24 hours immediately following. Warned thereby, we handled the tissues of the later animals with great circumspection and met no other instances of the sort.

The bile taken as a type sample of the liver output was collected into a rubber balloon which was connected with the common duct by a short cannula and a soft rubber tube 4 to 8 cm. long and of about 2 mm. bore. The cannula was bound down into line with the duct—which was merely slit, not severed—by a thread about its shank and the lower duct portion, and thus obstruction from a kink or elbow was rendered unlikely. The balloon was left within the peritoneal cavity, and the abdominal wall closed completely in three layers. Asepsis was maintained throughout. The dogs bore the operation well, and remained in excellent condition throughout the term of experiment which was usually 24



hours. Often the animals ate largely soon after operation. All were ultimately chloroformed and immediately autopsied. Cultures were taken of the bile specimens, and pieces of liver from the regions separately drained were placed on agar and in bouillon. Infection was rare, despite the fact that the bile accumulated at body heat. Its occurrence is noted in the tables.

The pigment content of the bile was used as the index to concentration by the bladder. The sojourn of the bile for some hours at the temperature of the animal was found by repeated *in vitro* test to be without effect on the pigment, save for the conversion of a negligible portion into biliverdin when air was present. To prevent this change it was only necessary to deflate completely the collecting balloons prior to their introduction. Routine colorimetric estimations were made by Hooper and Whipple's<sup>5</sup> modification of the Salkowski method, whereby bilirubin and biliverdin are estimated together; but instead of a color wedge of artificial constitution, we have employed as a standard pure bilirubin, and in place of the Autenrieth instrument, a Duboscq micro-colorimeter. The bilirubin (Schuchardt) was in chloroform solution, 1 mg. to every 4 cc.; and, to prepare a standard, 1 cc. of this was made up in a volumetric flask to 10 cc. with Hooper and Whipple's acid alcohol, and allowed to stand 18 to 24 hours at room temperature, when the characteristic blue-green color described by these authors was found to have developed. The biles were treated likewise save that 1 cc. was made to 50 cc. with the alcohol. The bladder contents was often so concentrated as to necessitate some preliminary dilution with water.

Bile treated with the acid alcohol did not always go through the same color changes. Often the tint ultimately developed for the readings tended somewhat to the green, as compared with the standard, or again was pronouncedly more blue. The normal bladder bile removed at operation frequently yielded a gamut of purples, possibly as a result of the presence of bilicyanin,<sup>6</sup> and could at no time be read against the standard. This never happened with specimens obtained after operation. Fortunately for our work, the biles obtained from different portions of one liver during the same period always went through identical color changes and in consequence could be accurately compared; while such errors in quantitation as were involved in reading them against a standard of different tint were applicable to them in like degree. The results given in our tables are expressed in milligrams of bilirubin save for six early instances, in which the standard proved faulty. For them "color units" are employed instead. But "color units" could have been used to report the findings throughout, since the main significance of these latter lies in relative not in actual pigment quantity.

For a standard in the early experiments, a chloroform solution containing only 5 mg. of bilirubin per 100 cc. was employed, the stock solution advocated

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<sup>5</sup> Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

<sup>6</sup> Hoppe-Seyler, F., in Hirschwald, A., *Handbuch der physiologisch- und pathologisch-chemischen Analyse, für Aerzte und Studierende*, Berlin, 8th edition, 1909, 371.

by van der Bergh and Snapper<sup>7</sup> in connection with the diazo reaction. A measured portion was dried on the water bath and the residual pigment taken up in acid alcohol. But it dissolved with a troublesome slowness, and furthermore the chloroform solution itself, after a month or two at room temperature, became greenish and much weaker as shown by the lessening color response with both acid alcohol and the diazo reagent.<sup>8</sup> With a concentrated stock solution having 1 mg. for every 4 cc., and kept in the ice box, the loss of pigment within 2 months is negligible. It can be followed in the colorimeter by a comparison of the acid alcohol standard made from it with a mixture of 10 cc. of copper sulfate solution (10 gm. to 100 cc. of water) and 0.075 cc. of potassium bichromate solution (1 gm. in 100 cc. of water). Indeed, an inorganic standard of the sort described will probably prove best in the long run for routine colorimetric purposes. Slight alterations in the amount of bichromate suffice to turn the color toward the blue or green without essentially altering its value.

### *Control Observations.*

The plan just outlined could be useful for our project only on the assumption that the bile from different liver regions has approximately the same pigment content per cubic centimeter. This cannot be taken as a foregone conclusion, if for no other reason than because the individual liver lobes receive portal blood from different visceral sources as a number of workers have shown.<sup>9</sup> To determine the actual case was our first step.

In six animals the neck of the gall bladder was tied off and the contents of the organ was removed by aspiration. A partitioning ligature was then placed upon the main duct as usual and, just above, a cannula connecting with a rubber balloon was inserted for collection of the bile that in later experiments was to flow instead to the bladder. The usual sample bag was then connected with the common duct and the laparotomy wound closed. Some of the animals were full fed at the time of operation, while others had fasted for 24 hours. All had access to food afterwards, but in general only those took it within the first 24 hours that had previously been denied. The factor was without notable influence on the result, as the table shows.

The liver regions supplying the two bags varied considerably from animal to animal because of differences in the duct arrangement. In two of the six instances more tissue was tributary to the sample bag than to the upper one.

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<sup>7</sup> van der Bergh, A. A. H., and Snapper, J., *Deutsch. Arch. klin. Med.*, 1913, cx, 540.

<sup>8</sup> For a note on the changes in bilirubin kept in chloroform see Oppenheimer, C., in Fischer, G., *Handbuch der Biochemie*, Jena, 1909, i, 731.

<sup>9</sup> Bartlett, F. K., Corper, H. J., and Long, E. R., *Am. J. Physiol.*, 1914, xxxv, 36.

The table of results (Table I) has been arranged with reference to the question whether the bile in the sample bag gives any index to the pigment concentration of that elaborated by the rest of the liver. It will be seen that this question is answered in the affirmative. The "bile strength," that is to say the pigment per cubic centimeter, is nearly the same for both portions of secretion, despite wide variations from animal to animal in the total fluid output and its bilirubin content (compare Experiments 2, 4, and 5). This is a weighty point for it means that in the later work the bile strength can be used to gauge the concentration effected by the gall bladder.

The actual fluid in the upper bag and the calculated amount as determined from a knowledge of the quantity in the lower, or sample, bag and the relative pigment content of the two, assuming that there was an equal degree of concentration, differ but little, as would follow from the circumstance that the actual bile strengths of both proved to be nearly identical. A calculation of the sort was regularly employed in the later work to gain an idea of how nearly the real amount of fluid reaching the gall bladder approximated that which should have reached it, judging from the partition of the hepatic tissue.

Some marked discrepancies will be noted between the actual fluid in the upper bag and the theoretical quantity as worked out from the partition of tissue. The effect of the partitioning ligature could not always be exactly ascertained, as for example, when a single lobe possessed ducts draining to either side of it. But this will not suffice to explain certain cases. In Experiment 6 the rubber tube connecting said bag with the duct, being stiffer than ordinary, was at autopsy found sprung like a bow, with one end pressed upon the neighboring portal trunk and thus perhaps diverting blood to the region tributary to the lower bag with result in more active secretion there. Whatever the cause of the other discrepancies these had no significance for our project.

The fact may be noted in passing that four animals out of five, with the instance of Experiment 6 excluded, yielded to the upper bag a bile that was relatively, if slightly, richer in pigment per cubic centimeter. This was no accident, as will be shown further on. There too, in Tables IV and V, are data that indirectly corroborate Table I.

*Concentrating Power of the Emptied Gall Bladder.*

Observations on the bladder were now begun. In a first series of experiments the organ was washed with salt solution, and left to fill with bile as it normally might fill, assuming that it empties on normal contraction.

A small slit was cut in the main bile duct at the point where the partitioning ligature was later to be laid on, and, through a silk catheter thrust up into the neck of the gall bladder, all bile was withdrawn and the organ washed with 0.9 per cent salt solution until the rinsings came away uncolored. Due care was taken to avoid overdistention, and the final emptying was accomplished by gentle pressure with moist sponges. The catheter was then withdrawn and ligatures were placed on the duct above and below the slit, to close it and divert the bile, part as usual into a bag, and the remainder to the empty bladder.

It may be asked whether the brief washing was effective; for a concentrated residuum of bile such as might be left clinging next the bladder mucosa would certainly complicate the findings. No difficulty was experienced on this score. The wall of the bladder is translucent, with the color of the contents shining through, and the efficacy of the washing can be controlled by direct inspection. Furthermore, the original bile was retained for comparison with that accumulating later in the sample bag. Practically always it was weak in pigment, relatively speaking,—whence one may conclude that any remnant of bladder bile left after the washing would act, if anything, to dilute in this respect the fluid entering later.

The contents at autopsy of the gall bladder was always so very viscid as well as dark that it was removed by rinsing with distilled water, and still further diluted prior to the withdrawal of a type portion. On opening the animal a clamp was laid on the bladder neck to prevent any passage of secretion from the hepatic duct. As a guide in the dilution, the assumption was made that as much bile had originally reached the gall bladder as was called for theoretically from the quantity in the sample bag and the proportion of hepatic tissue supplying the two; and the actual bladder contents was brought up to this amount. The mixture of bile and water was shaken repeatedly, allowed to stand several hours, and shaken again prior to the removal of 1 cc. for treatment with acid alcohol and comparison with a standard. The results with duplicate specimens showed that an even distribution of pigment had been brought about.

In all save one of this series of experiments, and in Experiment 6 of Table I, pigment values are expressed in "color units," the unit being the amount of pigment in 1 cc. of bile from the sample bag.

The period of experiment ranged in the five animals from  $22\frac{1}{2}$  to 49 hours. To our great surprise the gall bladder even at the end

of the longest period held but a few cubic centimeters of bile, far less than the amount necessary for normal distention; while after 24 hours, the organ was practically collapsed in two out of three instances, yielding only 0.77 and 1.4 cc. of fluid, and in the third case was but half full. Could one suppose that the hepatic tissue tributary to the bladder had failed to secrete as usual? Or had its output undergone concentration to an extent commensurate with the findings? The latter proved to be the case. The dark, syrupy or tarry, bladder contents had from 3.18 to 10.8 times the pigment strength of the fluid in the sample bag, with an average of 7.1 (Table II). The contents of the tributary ducts was always by contrast thin and weak in pigment, like the bag bile. There were only a few drops to be had from the ducts, too little for colorimeter readings, so dilutions with water were compared directly with similar dilutions of bag and bladder biles. Sometimes they were in addition tested for cholates by Hay's method. The results confirmed the pigment findings.

#### *The Concentrating Power of Full Gall Bladders.*

With the gall bladder emptied as in these experiments its whole concentrating influence is brought to bear upon the secretion arriving little by little, from the liver. In this favorable circumstance lay not impossibly one cause of our results. To determine the real case a series of animals was studied, of which the bladders were filled with bile of known pigment content, before the wash catheter was withdrawn.

The dog bile to be introduced was collected in bags under aseptic conditions and kept on ice for periods up to 48 hours. In one instance, that of the material derived from the upper bag of Experiment 5, Table I, it proved to have been infected with a micrococcus of dubious pathogenicity, and this organism was recovered in pure culture from the gall bladder into which the bile was put (Experiment 1, Table III). Less concentration was effected by the bladder in this instance than in any other of the series.

To fill the bladder under a known pressure while preventing the escape of any fluid into the peritoneal cavity, a ligature was placed upon the duct containing the catheter just above the slit in its wall, and this was tied down as the catheter was withdrawn, thus becoming one of the partitioning ligatures. The catheter itself was connected with a sterile funnel containing the bile. The pressure of a column of bile 60 to 100 mm. high proved just sufficient to effect a

normal distention of the bladder. This is less than the pressure withstood by the sphincter of Oddi.<sup>10</sup> Needless to say, the tributary ducts shared the pressure conditions. In Experiment 7, a pressure such as develops upon duct obstruction<sup>11</sup>—300 mm.—was used.

Our expectation was to find at autopsy a marked stasis with dilatation of all the passages above the partitioning ligature, owing to secretion into them of more bile than the gall bladder could cope with. But the event was quite another. So rapidly was fluid withdrawn through the bladder wall that the increments of hepatic secretion proved insufficient in most instances to hold the organ distended. It was found nearly collapsed in Experiments 1, 4 and, 7, while in Experiment 6, in which alone a normal distention was observed, its capacity was unusually small, only 3.5 cc. The amount of bile introduced at operation and, to a less extent, that removed furnished for each case an approximate measure of capacity.

The inspissating activity of gall bladders left distended (Table III) proved to be little behind that of emptied ones (Table II). On the average the concentration of pigment was 6.4 times that of the bag samples, with a range from 3.6 to 8.9. The bile collected from the tributary ducts was thin with relatively little pigment, showing, as in the animals with emptied bladders, that the secretion had not been elaborated in condensed form. The greatest concentrations—to bile strengths, 8.1 and 8.9 times that of the bag samples—were effected during only 18 and 22 hours respectively. The amount of secretion acted upon in these instances as calculated from the quantities of bilirubin in bag and bladder and the fluid content of the former,—assuming both bile portions to have had the same pigment strength originally,—was for the first case 59.8 cc. which was reduced to 7.4 cc. by an organ of 9 cc. capacity, and for the second 26.6 cc. brought down to 3 cc. by a bladder holding at most 4.3 cc.

In Experiments 3 and 4, the bile introduced at operation had previously been concentrated 3.5 times—it was the upper bag contents of Experiment 2, Table V—and was syrupy with mucus. These changes did not prevent a further inspissation in the bladder to pigment strengths of 4 and 5.9 times respectively that of the bag samples.

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<sup>10</sup> Judd, E. S., and Mann, F. C., *Surg., Gynec. and Obst.*, 1917, xxiv, 437.

<sup>11</sup> Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

In both Tables II and III it will be noted that the pigment found in the bladder frequently fell far short of the expected quantity (Table II, Experiments 4 and 5; Table III, Experiments 1, 3, 4, and 7). Must one suppose that part of the bilirubin reaching the organ had passed out through its wall, or was there diminished secretion above the partitioning ligature? Certainly pigment can pass the mucosa, for it has been observed histologically in transit;<sup>12</sup> but our observations upon the lymph indicate that the quantity thus removed is negligible. There is usually to be found coursing down the neck of the dog's gall bladder, and draining most of its extrahepatic portion, a large, turgid lymphatic yielding fluid in quantity when cut. We have frequently examined such fluid from gall bladders that held heavily pigmented bile. It was always practically colorless and failed to give positive reactions for bilirubin, though occasionally cholates were present, as shown by Hay's test. The direct passage of pigment into the blood stream cannot be ruled out, but it seems unlikely in view of these findings.

There remains the alternative of locally diminished secretion. The conditions in some cases would seem to have been highly favorable to this. The gall bladder in these instances was found at autopsy to be nearly collapsed, and so bound down by fresh adhesions between the adjacent liver lobes that its redistension could scarcely have been brought about by the normal secretory pressure. Under these circumstances, there may well have occurred a stasis in the tributary ducts at periods when secretion by the liver provided more fluid than the bladder could immediately concentrate. Direct proof of this was not obtainable because the necessary observations involved a severing of the very adhesions whereby the abnormal state was maintained. But it is interesting to note that the total output of bile pigment per kilo of animal averaged precisely the same (11.1 mg. in 24 hours, a normal amount) for the dogs of Tables II and III with a relatively small yield above the partitioning ligature, as for those of Tables I, IV, and V in which this was not the case. The fact suggests that whatever the cause of the small yield above the ligature, it resulted merely in a shift of the secretory activity with an unduly large output to the sample bag, and, by corollary, unwarranted expectations as to what should have been provided to the gall bladder.

#### *The Bladder Utilized as a Duct.*

The partitioning ligature of the preceding experiments could not be relaxed like the sphincter of Oddi. Fluid remained pent above it no matter how powerfully the gall bladder may have contracted. Here was an important departure from the normal; and the question arises whether the removal of fluid from the bile may not have been largely the consequence of pressure intermittently exerted by the bladder wall. For such reason another series of experiments was

<sup>12</sup> Aschoff, L., and Bacmeister, A., *Die Cholelithiasis*, Jena, 1909.



performed in which a partitioning ligature and sample bag were placed as usual, but the free tip of the bladder was connected by cannula with a second bag.

The tip of the bladder was seized with hemostats and a small slit made at the point where blood and lymph vessels were least abundant. The bile was removed by aspiration, and the usual flushing with salt solution; and a glass cannula with trumpet mouth and a least inside diameter of 2 to 3 mm. was fixed in place with a purse string suture. A rubber tube of the same bore, 4 to 8 cm. long, led from it to the rubber balloon. At autopsy this tube was clamped off as soon as the peritoneal cavity had been opened, to prevent the shifting of bile in either direction.

Four dogs were operated upon. The results are given in Table IV. The new cystic outlet was of somewhat larger caliber than the normal one, and at the most dependent portion of the bladder which was found practically empty at autopsy. The bile, urged by the secretory pressure and by gravity, had evidently run directly through, as through any other channel to the bag, being aided in two instances by a postoperative drawing together of the bladder wall which had much narrowed and shortened the organ. Nevertheless, the bile that had been submitted to it proved to be 2.3 to 4.8 times as concentrated as that in the corresponding sample bag. In view of such findings the results of Tables II and III cannot be attributed to the closed system existing above the partitioning ligature.

In the animals of Table IV the pigment content of the upper bag differed but slightly from the theoretical amount, as calculated from the quantity in the sample bag and the proportion of tissue tributary to each. In this respect the findings were nearer perfection than in the control series of Table I. The absence of any cause for local portal obstruction such as was provided by the upper cannula in the dogs of Table I may have been responsible for this.

In final illustration of the concentrating activity, three experiments originally intended as controls will be reported, which were carried out prior to realization of the bladder capabilities. To obtain the bile from above the partitioning ligature in these instances, a cannula of large bore was thrust through a cut in the bladder wall into the neck of the organ and secured there. The duct from the right side of the right central lobe enters so high up that there is often no true cystic duct, and in order to avoid obstruction of this



tributary, the cannula was not pushed down to its level but left with a tiny pouch of bladder mucosa about its mouth. That the influence of the pouch was far from negligible is shown by the results. The upper bag yielded a bile syrupy with mucus and considerably richer in pigment per cubic centimeter than the thin, sample fluid (Table V).

It may be recalled that in the controls of Table I the bile from the upper bags was generally slightly the more concentrated. Now it so happens that in the dog the gall bladder wall extends some distance down the duct, the macroscopic character of the latter being often first evident below the entrance of the highest branch from the liver. Others have noted this before us. Indeed, both in the dog and in man a new gall bladder may develop out of the remnant of bladder mucosa left by a cholecystectomy that has failed to include the cystic duct.<sup>13</sup> It follows that a ligature placed on the neck of the bladder to block the organ off, as in the experiments of Table I, may frequently fail in some part of its function. To such a happening do we attribute the slightly greater concentration of most of the upper biles of Table I. For, as Table IV demonstrates, a transient exposure of the bile to but a fraction of the bladder wall results in a reduction of its bulk.

#### *Influences of the Ducts.*

For the purposes of the present study the duct system proper has been deemed without influence upon the fluid it conveys. But in view of the great activity of the gall bladder, is such an assumption warranted? For practical purposes it is, as we shall show in an accompanying paper. The ducts instead of withdrawing fluid from the bile tend to dilute it slightly with a watery product of their own.

#### *Peculiar Character of the Bile Acted upon.*

No such deeply pigmented biles as the gall bladder yielded at the end of our experiments are found under normal conditions. Normal bladder bile of the dog is often light yellow, and, at most, of a

<sup>13</sup> Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 710. Haberer, H., and Clairmont, P., *Verhandl. deutsch. Ges. Chir.*, 1904, xxxiii, pt. 2, 81.

medium brown tint, whereas that now referred to was always dark, and frequently brown-black. It may be recalled that the activities of the bladder had been brought to bear on but a fraction, and sometimes a small fraction, of the total secretion. But this is not the sum of the matter. For quantitation of the bag samples showed clearly that the liver had furnished an abnormal secretion, one extremely rich in pigment and small of bulk.

The bile of healthy dogs has ordinarily from  $\frac{1}{3}$  to  $\frac{1}{2}$  mg. of bilirubin in every cc., and practically never as much as 1 mg.;<sup>14</sup> whereas that of our animals contained after operation more than 2 mg. per cc. usually, only once less than 1 mg., and in one instance 6.8 mg. Such plenitude was attained almost wholly at the expense of the fluid output, as shown both by direct measurement of the latter and by the fact that the total pigment elaborated by the liver in the 24 hours immediately after operation was, if anything, only a little increased over the normal. The day to day output of bile varies greatly, a fact that Stadelmann<sup>15</sup> has emphasized. He gives figures for two dogs weighing 16 to 17 kilos which show that they secreted about 288 cc. of bile per 24 hours, that is to say slightly more than  $\frac{3}{4}$  cc. per kilo in 1 hour. But the six dogs of our Table I yielded respectively  $\frac{1}{18}$ ,  $\frac{1}{11}$ ,  $\frac{1}{3}$ ,  $\frac{1}{4}$ ,  $\frac{1}{10}$ , and  $\frac{1}{3}$  cc. per postoperative hour, amounts that are with one exception greatly below Stadelmann's average. The more indirect data of the other tables confirm the point thus illustrated. According to Stadelmann and Hooper and Whipple,<sup>5</sup> the normal bilirubin output is about 1 mg. per pound of dog in 6 hours, or for present purposes 8.8 mg. per kilo of animal in 24 hours. In our animals the pigment put forth after operation ranged from 8 to 13.4 mg. per kilo in 24 hours in uncomplicated cases, with an average of 11.1 mg. The two complicated cases (Experiments 7 of Table III and 5 of Table II) that were left from this computation yielded 7.3 and 15.4 mg. respectively.

One cause for the peculiar character of the postoperative bile at once suggests itself. Fasting animals, as is well known, yield but little bile, and this heavily pigmented and with a high percentage of solids.

In a dog followed by Stadelmann the secretion of the first 24 hours after food was withdrawn had a bulk only half that in the period immediately previous and in a second 24 hours less than one-third. The total pigment output, though,

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<sup>14</sup> The pigment studies of Hooper and Whipple with fistula animals may be consulted upon this point.

<sup>15</sup> Stadelmann, E., *Der Icterus und seiner verschiedenen Formen*, Stuttgart, 1891.

underwent no change. The observation will explain many, perhaps all, of our instances. The food intake of our dogs and their water intake as well, in the preliminary and experimental periods together, was far below the normal. Nearly all of the animals that took food in the 24 hours following operation had fasted through a like period prior to it.

### *Nature of the Concentrating Faculty.*

It would be highly interesting to know the exact composition of the scanty, dark, postoperative liver bile. For the withdrawal of fluid from it in the gall bladder is accomplished entirely through osmosis and diffusion, and the concentration thus achieved will necessarily vary with the bile character.

Brand<sup>2</sup> has found that both the hepatic and bladder biles have the  $\Delta$  of the blood; and through comparative analyses he obtained clear evidence that the concentration effected in the bladder comes about at the expense of the inorganic salts which are removed as what is, practically speaking, a normal saline solution. Hammarsten<sup>3</sup> and others confirm this, in that they too find a less quantity of inorganic salts in bladder bile than in liver bile, but correspondingly more of substances having large molecules.

The fact that the limit of biliary concentration is the  $\Delta$  of the blood will explain our observation that the gall bladder contents showed no greater degree of inspissation after 48 hours (Experiments 3 and 4, Table II) than after one-third to one-half this period (Experiments 1 and 2, Table II, and all of Table III). The shifting of constituents whereby a reduction of the bile volume comes about takes place so rapidly (Table IV) that it must be practically complete within a few hours. In the lack of comprehensive analyses, the suitability of the heavy, postoperative liver bile of our experiments for concentration in the gall bladder cannot be profitably discussed. But there is every reason to suppose that its limit of concentration would be reached far sooner than that of the normal secretion, which is, by contrast, watery.

Under the operative conditions we have employed, some obstruction to the passage of fluid away from the bladder must not infrequently have been caused. The large lymphatics derived from the viscus course close beside the main duct in the gastrohepatic omentum, and the slight dissection required to place the partitioning

ligature, together with the pull of the latter, cannot but have sometimes compromised such delicate vessels. As offsetting this in that it favored resorption of fluid may be put the preliminary cleansing of the gall bladder mucosa with salt solution. But in the course of many observations upon normal dogs we have only rarely encountered a mucous layer next the bladder wall. Usually the organ holds a thin, practically homogeneous syrup, and the mucosa is clean. That a moderate mucus admixture need not greatly hinder concentration is shown by Experiments 3 and 4 of Table III with a secretion already rendered syrupy and more than thrice concentrated by the gall bladder of another animal. Taken as a whole, the conditions of our experiments were probably rather unfavorable to the concentration of bile.

#### *Bladder Fistulæ.*

Practically all quantitative studies of the bile from day to day have been carried out on fistula animals, with the gall bladder as a link in the fistulous system. The bile runs from the ligated common duct through the gall bladder and out by a slit in its tip which is sewn fast to an opening in the abdominal wall. Our findings of Table IV show that this practice involves great possibilities of error as regards actual bulk of the liver secretion, since it may be much reduced in transit. Perhaps, though, the bladder wall soon loses its concentrating faculty, owing to pathological change. Instances have been described of temporary obstruction to a fistulous outlet in which the bladder was found filled, not with inspissated bile, but with hydropic fluid,<sup>16</sup> the product of a damaged mucosa.<sup>12</sup> Nevertheless, the possibility of concentration by the bladder must be kept in mind in reviewing the data of fistula experiments.

#### *Functions of the Gall Bladder.*

There appears to be little general realization of the physiological uses of the healthy gall bladder which has now become a favorite surgical trophy. Yet several attested purposes the organ has.

<sup>16</sup> Kölliker, A., and Müller, H., *Verhandl. physik.-med. Ges. Würzburg*, 1856, vi, 435.

Pawlow's assistants<sup>17</sup> have shown that an intermittent discharge of bile takes place into the duodenum during the passage of chyme from the stomach, but ceases with this. Thereafter, until food is again taken, only a very occasional spurt of secretion passes the sphincter of Oddi—about once an hour in the dog.<sup>13</sup> The first bile expelled into the chyme is recognizably bladder bile, being syrupy and usually darker than that coming later, though both escape under pressure, in small spurts or jets, at short intervals. After cholecystectomy a great difference is observed. Bile dribbles continuously from the ampulla of Vater<sup>13</sup> and during fasting may fill the duodenum and be voided as such in the stools.<sup>13</sup> The disturbance of function thus indicated is not without a bad effect on the digestive processes,<sup>18</sup> masked though this usually is; and Rost has described a striking anatomical change that is common in man and the dog after cholecystectomy; viz., a general dilatation of the bile passages. His finding has been confirmed by numerous observers. The dilatation does not occur when the sphincter of Oddi is destroyed.<sup>10</sup> Rost applies the term "biliary incontinence" to the continuous escape of secretion into the intestine after removal of the gall bladder. The incontinence is associated with an abnormal relaxation of the sphincter<sup>10</sup> which latter, however, frequently recovers its tone as duct dilatation ensues.<sup>13</sup>

Such activities as are more or less directly illustrated by these facts fall into three categories. The gall bladder acts like a distensible bag interpolated into a rigid system of tubes, to minimize extremes of pressure when bile comes rapidly or in large quantity from the liver and its escape into the intestine is prevented by the sphincter. The bag in question is rendered capacious not so much through its size as by a singular ability to reduce the bulk of the fluid reaching it. Small wonder that after cholecystectomy the ducts dilate and the sphincter gives way!<sup>19</sup> The organ is also propulsive, delivering bile to the duodenum when needed. But such service is subsidiary, if essential, to the storage of bile. During those periods when the duodenum is empty the bladder husbands the bile for future use, and through its concentrating activity is enabled to retain very nearly all of the liver output when the interval from one gastric digestion to another is not unduly long. An illustration of the point may be given:

<sup>17</sup> Bruno, G. G., Dissertation, St. Petersburg, 1898, and Klodnizki, Dissertation, St. Petersburg, 1898; cited by Babkin, B. P., *Die äussere Sekretion der Verdauungsdrüsen*, Berlin, 1914, 344. Bruno, G. G., *Arch. Sc. biol. St. Pétersbourg*, 1899, vii, 87.

<sup>18</sup> Hohlweg, H., *Deutsch. Arch. klin. Med.*, 1912, cviii, 255.

<sup>19</sup> Judd, E. S., *Ann. Surg.*, 1918, lxxvii, 473.

The period of most abundant bile formation in a normal animal coincides roughly with that during which chyme leaves the stomach. Secretion has diminished markedly 10 or 12 hours after the ingestion of food,<sup>15</sup> and thereafter continues slowly to lessen. A normal dog of 9 kilos fed every 12 hours, that is to say under favorable conditions for secretion, will form, in every 12, about 90 cc. of bile.<sup>20</sup> Much less will be put out when a feeding is omitted. Now if such a dog be supposed to have a gall bladder holding 10 cc., which is well below the average capacity,<sup>21</sup> and the organ be endowed with the ability to concentrate the bile sixfold, which in view of our experimental findings is not too much to assume, there should be room in it for 60 cc. of liver bile. That so much will actually come from the liver is doubtful. The hourly small spurt into the duodenum during a fast is not indicative of a tensely filled gall bladder but may be directed to the maintenance of sterility of the passages.

In all likelihood the gall bladder has functions in addition to those outlined. Its importance as a reservoir is perhaps less in animals that, like man, eat frequently, than in species such as the dog that habitually go long periods without food. There may be reasons for the concentrating activity besides the reduction in fluid bulk. And the need for mucus in the bile is unexplained. The elaboration of mucus in quantity is, like the concentrating activity, a function of the bladder as distinct from the ducts. Indeed, the receptaculum chyli has been much too often considered a mere diverticulum in the duct system. The special character of its influence upon the bile deserves emphasis as demonstrating the highly purposeful differentiation of the organ. The fact that few ills follow upon removal of the normal gall bladder means merely that the body has adapted itself to the loss, not that the loss is unimportant. In this connection the surgeon would do well to remember that uncertainty as to function and confidence in readjustment are at best questionable motives for adventures in ablation.

<sup>20</sup> To judge from Stadelmann's instances.

<sup>21</sup> According to Mann (Mann, F. C., *New Orleans Med. and Surg. J.*, 1918, lxxi, 80), the average capacity for a dog of 8 kilos is 16.6 cc. The individual variation is great, as our Table III shows. Mann's figure indicates that we had to do with unusually small bladders, a view supported by our more recent experience.

## SUMMARY.

The bile coming at one time from different portions of the liver of the dog has nearly the same amount of pigment per cubic centimeter. With this determined we have studied the power of the gall bladder to concentrate bile directed to it, using as criterion the pigment strength of a sample collected throughout the period of experiment from a duct branch. The extent and rapidity of the concentration are alike remarkable. A gall bladder emptied at the beginning of one experiment and left to fill from the liver, concentrated the 49.8 cc. of bile reaching it in  $22\frac{1}{2}$  hours to 4.6 cc., that is to say reduced its bulk 10.8 times; while another bladder left distended with a bile of known constitution and receiving in addition fresh increments from the liver concentrated the secretion 8.9 times in 22 hours. A series of five emptied bladders concentrated the bile coming to them in about 24 hours on the average 7.1 times, or a little more than the 6.4 times of seven organs left full. The conditions in both cases were relatively unfavorable to the withdrawal of fluid from the bile because this takes place by osmosis and diffusion, with the ultimate  $\Delta$  always that of the blood, and the secretion in our animals was notably rich in solids as an indirect result of the operation.

The rapidity with which fluid is withdrawn through the wall of the bladder may be judged from some experiments in which a bag was connected with the tip of the organ by a large cannula. Merely in its passage through the bladder the bile was concentrated 2.3 to 4.8 times. The finding indicates a potential source of error in observations upon samples of bile obtained from fistulous channels of which the bladder forms a part.

The bile ducts do not withdraw fluid from the secretion they convey but tend to dilute it, as we shall show in a companion paper. The restriction of the concentrating activity to the receptaculum chyli is good evidence that the latter has special significance for the organism. The nature of this significance is briefly discussed.

TABLE I.  
*Control Instances.*

	Period.	Bag.	Lobes drained.	Weight of tissue drained.	Bile amount.				Pigment.			Bile strength.	Remarks.
					As calculated from		Actual.	Calculated total.	Actual total.	Amount per cc.			
					Tissue.	Pigment output.							
											cc.		
Experiment 1; ♂; 12¾ kilos (fasting).	hrs. 24	Sample.	Right lateral and caudate.	gm. 154.0	cc.	cc.	cc.	cc.	mg.	mg.	mg.	1	About 74 gm. of tissue in right side of right central lobe totally obstructed by the ligature; partition of tissue only approximately known.
	Upper.	Left central and lateral; papillary; left side of right central.	244.0	11.4	9.5	9.9	77.5	64.5	6.5	0.96			
Experiment 2; ♀; 8 kilos (fasting).	24	Sample.	Right lateral and caudate.	91.5			7.6		25.0	3.3		1	Very early pregnancy.
	Upper.	Remainder of liver.	161.0	13.4	12.9	10.3	44.0	42.4	4.1	1.25			
Experiment 3; ♀; 6½ kilos (full fed).	24	Sample.	Left lateral and central.	105.0			27.3		48.1	1.76		1	
	Upper.	Remainder of liver.	130.7	34.0	26.8	23.3	60.0	47.2	2.0	1.15			



Experiment 4; ♀; 7 kilos (full fed).	24	Sample.	Left lateral, half of left cen- tral; caudate and right lat- eral.	138.0					30.0	1.1	1	Partition of tissue only approxi- mately known; very early preg- nancy.
		<i>Upper.</i>	Remainder of liver.	94.0	19.1	24.4	19.0	20.4	26.1	1.4	1.28	
Experiment 5; ♀; 9 kilos (some food).	17	Sample.	Right lateral, caudate, and two-thirds of left lateral.	154.0			70.0		70.0		1	Partition of tissue only approxi- mately known; pigment in units, not mg.; bile of upper bag in- fected.
		<i>Upper.</i>	Remainder of liver.	210.0	95.5	76.8	68.5	95.5	76.8		1.12	
Experiment 6; ♂; 8 kilos (fasting).	24	Sample.	Right and left lateral and caudate.	149.0			48.0		48.0		1	Pigment in units; probable inter- ference with portal flow to tissue drained by upper bag; both biles syrupy.
		<i>Upper.</i>	Remainder of liver.	105.0	33.8	15.5	19.0	33.8	15.5		0.82	

Some of the animals had received no food in the 24 hours prior to operation. The fact finds parenthetical record in the first column of the table. All of the animals had access to food afterwards, but in general only those ate that had fasted previously.

TABLE II.

*Emptied Gall Bladders.*

	Period.	Bile.	Lobes drained.	Weight of tissue drained.	Kind of bile.	Bile amount.			Pigment.			Bile strength.	Remarks.
	<i>hrs.</i>			<i>gm.</i>		As calculated from		Actual.	Calculated total.	Actual total (units).	Amount per cc.		
						Tissue.	Pigment output.	<i>cc.</i>	<i>mg.</i>		<i>mg.</i>		
Experiment 1; ♂; 8 kilos.	22½	<i>Bag.</i>	Right lateral and caudate.	77.0	Golden brown, thin.			19.1		19.1		1	Hay's test yields similar findings; gall bladder half collapsed; right pneumothorax.
		<i>Bladder.</i>	Remainder of liver.	202.0	Dark brown, syrupy.	50.2	49.8	4.6		49.8		10.8	
Experiment 2; ♀; 9¼ kilos.	24	<i>Bag.</i>	Right and left lateral; left central; caudate.	198.0	Dark brown, thin.			15.0		15.0		1	Hay's test yields similar findings; gall bladder collapsed.
		<i>Bladder.</i>	Right central; papillary.	90.0	Brown-black, tarry.	6.8	6.65	0.77		6.65		8.6	
Experiment 3; ♂; 11¼ kilos.	46½	<i>Bag.</i>	Caudate; right and left lateral and central.	296.0	Dark brown, thin.			47.5		47.5		1	Gall bladder half collapsed.
		<i>Bladder.</i>	Right central; papillary.	127.0	Brown-black, tarry.	20.6	22.8	4.9		22.8		4.65	

Experiment 4; ♂; 11 kilos.	49	Bag.	Left central and lateral lobes.	141.0	Dark brown, thin.				77.0		77.0	1	Gall bladder half collapsed.
		<i>Bladder.</i>	Right lateral and central; papillary; caudate.	196.0	Brown- black, tarry.	107.0	43.8	5.4	43.8			8.1	
Experiment 5; ♀; 4½ kilos.	24	Bag.	Right and left lateral; left central; caudate.	95.0	Dark brown, thin.			27.75	59.8	2.15		1	Probable stasis in ducts leading to gall bladder owing to adhe- sions which bind latter down; pig- ment in mg.
		<i>Bladder.</i>	Right central; papillary.	43.5	Dark brown, syrupy.	12.7	4.45	1.4	9.6	6.86	27.4	3.18	



Experiment 4; ♀; 8½ kilos.	25	Bag.	Right and left lateral and caudate. Remainder of liver.	188.0	Dark brown, thin. Very dark brown, viscid.	(29.5) 41.3	(13.4) 25.2	37.75	67.7	1.8	1	11.8 cc. of syrupy bile containing 26.7 mg. of pigment left in gall bladder at 65 mm. pres- sure.
		Bladder.		147.0				4.3	(53.0) 79.7	(24.1) 50.8	5.9	
Experiment 5; ♀; 6 kilos.	22	Bag.	Right lateral and cau- date. Remainder of liver.	105.0	Dark brown, thin. Very dark brown, viscid.			20.6	36.0	1.75	1	4.3 cc. of bile containing 5.4 mg. of pigment left in gall bladder at 70 mm. pressure.
		Bladder.		180.0		(35.2) 39.5	(22.3) 26.6	3.0	(61.7) 67.1	(39.0) 44.4	8.9	
Experiment 6; ♀; 6½ kilos.	24½	Bag.	Right lateral and cau- date. Remainder of liver.	59.0	Dark brown, thin. Brownish black, thick.			8.3	27.1	3.3	1	3.5 cc. of bile containing 7.1 mg. of pig- ment left in gall bladder at 90 mm. pres- sure.
		Bladder.		134.0		(18.8) 22.3	(20.0) 23.5	3.5	(61.6) 68.7	(65.4) 72.5	6.7	
Experiment 7; ♂; 7¼ kilos.	24	Bag.	Right lateral and cau- date only? Remainder of liver.	98.5	Greenish brown, thin. Dark brown, viscid.			33.5	47.9	1.4	1	At autopsy gall bladder bound down by fresh adhesions; 4.5 cc. of bile, con- taining 7.9 mg. of pigment had been left in it at 300 mm. pressure.
		Bladder.		163.0		(55.5) 60.0	(5.9) 10.4	1.4	(79.4) 87.3	(8.4) 16.3	7.4	

\* The figures in brackets = contribution of the liver to the gall bladder contents; those unbracketed = sum of this and of material introduced at time of operation.

TABLE IV.  
*Bladder Fistula.*

	Period.	Bag.	Lobes drained.	Weight of tissue drained.	Kind of bile.	Bile amount.			Pigment.			Bile strength.	Remarks.
						As calculated from	Actual.		Calculated total.	Actual total.	Amount per cc.		
	hrs.			gm.		Tissue.	Pigment output.		mg.	mg.	mg.		
Experiment 1; ♂; 12 kilos.	24	Control.	Right lateral and caudate.	98.0	Thin, medium brown.	cc.	cc.	cc.	mg.	42.1	2.4	1	Gall bladder flaccid, contains 0.3 cc. of bile; cannula from it 3 mm. in least diameter.
		Bladder.	Remainder of liver.	249.0	Syrupy, dark brown.	44.4	49.5	16.2	106.9	119.0	7.4	3.1	
Experiment 2; ♂; 9½ kilos.	24½	Control.	Right lateral and caudate.	118.0	Thin, medium brown.			10.75		23.6	2.2	1	Gall bladder shrunken, contains 1.4 cc. of bile pressed back into it at autopsy; cannula from it 3 mm. in least diameter.
		Bladder.	Remainder of liver.	255.0	Thin, dark brown.	23.2	25.7	11.2	51.0	56.3	5.0	2.3	

Experiment	9	Control.	Caudate and part of right lateral.	64.0	Thin, medium brown.	50.7	47.4	10.3	22.6	2.2	1	Partition of tissue approximate; gall bladder contains 0.6 cc. of deeply stained mucus; lower bag contents infected with an organism that does not alter the pigment; cannula 2.5 mm. in least diameter.
3; ♂; 15½ kilos.		<i>Bladder.</i>	Remainder of liver.	315.0	Ropy, brownish black.			9.8	103.9	10.6	4.8	
Experiment 4; ♂; 11½ kilos.	23½	<i>Control.</i>	Caudate and right lateral.	73.0	Thin, dark brown.			15.0	35.1	2.3	1	Gall bladder shrunken, empty; cannula 3 mm. in least diameter.
		<i>Bladder.</i>	Remainder of liver.	205.5	Syrupy, much darker brown.	42.3	41.1	10.25	96.1	9.4	4.1	

TABLE V.  
*Cannula in Bladder Neck.*

	Period.	Bag.	Lobes drained.	Weight of tissue drained.	Kind of bile.	Bile amount.			Pigment.			Bile strength.	Remarks.
						As calculated from		Actual.	Calculated total.	Actual total.	Amount per cc.		
	hrs.			gm.		Tissue.	Pigment output.	cc.	mg.	mg.	mg.		
Experiment 1; ♂; 11½ kilos (full fed).	20	Lower.	Right lateral and caudate.	200.0	Medium brown, thin.			43.0		43.0		1	Pigment in units, not mg.
		Upper.	Remainder of liver.	305.0	Similar color (thick ?).	65.6	56.5	40.0	65.6	56.5		1.41	
Experiment 2; ♂; 8 kilos (fasting).	21½	Lower.	Upper part of right lateral lobe.	43.0	Dark brown, thin.			5.2		3.4	0.66	1	Partition of tissue only approximately known. Stasis in ducts to up- per bag?
		Upper.	Remainder of liver.	316.0	Dark brown syrupy.	38.2	100.0	28.8	25.0	65.6	2.3	3.48	
Experiment 3; ♀; 10¼ kilos (fasting).	26	Lower.	Right lateral and caudate.	115.0	Dark brown, thin.			12.5		42.3	3.4	1	
		Upper.	Remainder of liver.	245.0	Dark brown, syrupy.	21.3	33.5	20.0	90.0	113.6	5.7	1.68	



*General Description of the Tables.*

The most important data given relate to the relative pigment concentrations, or bile strengths, of the portions of secretion derived from the different liver regions of the same animal, that of the so called sample bag being taken as a standard. Table I shows that when the influence of the gall bladder is ruled out, the portions differ little in pigment value. The other tables present evidence that this value undergoes a manifold increase in bile submitted to the bladder.

Since the pigment strength is much the same for the unmodified secretion from different portions of a single liver, it becomes possible to ascertain approximately the quantity of bile reaching a gall bladder during a given period from the pigment accumulation in it as compared with that in a type specimen of the liver bile collected into a bag. Thus, for example, in Experiment 1 of Table II the bladder contained 49.8 units of pigment (a unit = pigment in 1 cc. of sample bile) in only 4.6 cc. of fluid, as compared with 19.1 units in the 19.1 cc. of the type specimen. It follows that 49.8 cc. of secretion had reached the bladder. Data obtained in this way have been given place in the tables. So too have figures on bile output and pigment quantity derived from a knowledge of the contents of the sample bag and the proportion of tissue tributary to it and to the bladder. In Experiment 1 of Table II the output of 77 gm. of liver, as collected in the sample bag, amounted to 19.1 cc., while 202 gm. supplied the bladder. It follows that the latter should have received 50.2 cc. of bile, an amount closely approximating the real one (49.8 cc.) as calculated out on the basis of actual pigment content. For the purposes of a comparison with this last, the total pigment that should theoretically have reached the bladder has been calculated out on the basis of the tissue partition and the pigment in the sample bag.

In the experiments of Table III the use of a foreign bile to distend the bladder has complicated the expression of results. To determine the amount of secretion and of pigment coming from the liver during the experiment, it was necessary to deduct from the ultimate findings the amounts introduced. This has been done. The figures in brackets represent the amounts of bile and of pigment furnished by the liver, and the unbracketed figures just beneath represent the sum of such quantities and of those introduced. Thus in Experiment 1, 10.3 mg. of pigment was put in the gall bladder and 14.2 mg. found at the close of the experiment. The liver then had contributed 3.9 mg. of pigment, or 1.6 cc. of fluid, judging from the pigment strength of that in the sample bag. Since 10.5 cc. of fluid had originally been introduced, the total acted upon by the gall bladder was 11.9 cc., and in the reduction of this to 3.3 cc. a 3.6 fold concentration was effected.



# PHYSIOLOGICAL CAUSES FOR THE VARIED CHARACTER OF STASIS BILE.

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PLATE 4.

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The fluid encountered at operation in the obstructed bile passages of human beings is of notably various character. Even in cases free of infection all gradations may be found between a black, tarry material and the watery, colorless "white bile" that has long puzzled surgeons. The causes for this diversity are not immediately evident in clinical instances because of the numerous complicating factors which give to each an almost individual peculiarity. One looks in vain for a clue to them in such a book as that of Kehr<sup>1</sup> which describes in detail the findings in many hundred operations upon diseased bile passages. But they are readily ascertained through experiment. The different, and in general opposed, functions of the gall bladder and ducts are principally responsible—infection aside—for the protean character of stasis bile.

## *Method.*

Dogs have been mostly employed for the work, with some cats and *rhesus* monkeys. Many animals used for concurrent observations on other themes were available. Obstruction to the bile ducts was produced by tying and cutting, with the excision, where possible, of a piece, and at difficult points by ligatures laid on in series. In dogs and cats the danger of a restoration of continuity by cutting through of the silk thread was found to be negligible when that used was of large caliber. The operations were performed aseptically under ether anesthesia, and the abdominal wound was closed in three layers. Infection and other complications were rare. Save where specifically mentioned, instances showing them have been ruled from consideration. After some days or weeks

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<sup>1</sup> Kehr, H., *Drei Jahre Gallensteinchirurgie. Bericht über 312 Laparotomien am Gallensystem aus den Jahren 1904-06*, Munich, 1908.

the animals were chloroformed and the ducts, their contents, the liver, and the hepatic vessels were carefully studied. Bits of the tissue and drops of the stasis bile were placed on agar and in bouillon, and the hepatic tissue was examined histologically.

The common duct of the dog and cat is formed from three or more main hepatic branches, high up into one of which the gall bladder empties. The arrangement varies greatly from animal to animal, and by taking advantage of special instances one may obstruct every large duct in turn, now in connection with the gall bladder and again separately, in association with a total obstruction, or with a local one so small as to be insignificant for the organism. In this way it is possible to test whether any duct has functions peculiar to itself. There is, properly speaking, no cystic duct in the dog or cat, owing to the entrance just below the bladder neck of a tributary from the right side of the right central lobe, but in the monkey there is a slender one, which, like that of man, empties into the common duct, an arrangement which much limits the obstructive permutations possible to the experimenter.

When the material was sufficient, quantitative estimations were made of the pigment and cholates in the stasis bile. In our opinion the results so obtained are not to be accepted without reservation because bilirubin, at least, undergoes changes on incubation in the gall bladder;<sup>2</sup> but they suffice to indicate the trend of affairs. Hooper and Whipple's<sup>3</sup> modification of the Salkowski test was used to estimate pigment. The method has been discussed at some length in a preceding paper.<sup>4</sup> For bile salts, the Foster and Hooper's<sup>5</sup> amino nitrogen method was employed when the material was sufficient, and Hay's sulfur test when it was not. The amino-acid determinations were carried out for us in Dr. Van Slyke's laboratory. The Pettenkofer test for bile salts could not be used because it yields a positive result with cholesterol. To disclose the presence of the latter the Liebermann-Burchard method was employed.

### *Contrasting Types of Stasis Bile.*

Whenever an obstructed bile duct was left in communication with the gall bladder the stasis bile later found proved to be heavily pigmented, and syrupy, ropy, or even tarry, according to whether the period of obstruction had been short or long.

*Experiment 1.*—In sixteen dogs, two cats, and one monkey obstruction of the common duct was produced, or of one or more of its hepatic tributaries in such wise that the gall bladder still communicated with the channels in stasis.

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<sup>2</sup> Hammarsten, O., *Lehrbuch der physiologischen Chemie*, Wiesbaden, 8th edition, 1914.

<sup>3</sup> Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

<sup>4</sup> Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

<sup>5</sup> Foster, M. G., and Hooper, C. W., *J. Biol. Chem.*, 1919, xxxviii, 355.

The changes that took place in the stasis bile were the same, whether total obstruction had been produced or not. In animals killed after 2 to 4 days the gall bladder and the ligated ducts connecting with it were tensely, though not greatly, distended with a syrupy, dark brown bile. 2 or 3 days later the color of the fluid was noted to be definitely green-brown, and after 10 days to 2 weeks of stasis it was a green-black, and the contents of the gall bladder was mucinous or even tarry, while that of the connecting ducts was a heavy syrup. In the next 3 or 4 weeks the bladder bile underwent no important change but the fluid in the ducts gradually thickened to a jelly which gave Hay's reaction for bile salts in high dilution. Further our observations did not go.

The contents of ducts separately ligated and of the obstructed common duct blocked off from the gall bladder was entirely different.

*Experiment 2.*—In nineteen dogs, three cats, and four monkeys, the common duct was obstructed and the gall bladder neck as well, or else one or more large hepatic ducts separately tied and cut.

The stasis fluid in these instances was at first brown, then green but definitely less pigmented, and finally, after 10 days or more of stasis, clear, often completely colorless, even in jaundiced animals, or at most of a pale yellow, and usually without sufficient cholates to give Hay's or Udranszky's test. That from the cat was syrupy and in one case a tenacious jelly, but the yield of the dog and monkey was watery with a slight, translucent, glairy admixture. No greater contrast to the inspissated biles of Experiment 1 could have been devised. Yet both types of stasis fluid were frequently obtained at one time from the same animal (Figs. 1 and 2).

*Experiment 3.*—Obstructions were so placed on the bile channels of twenty-one dogs, three cats, and two monkeys that some of the large ducts in stasis were deprived of their connection with the gall bladder, while others still possessed it.

At autopsy both sets of ducts were equally distended, the one with a heavy, green bile giving Hay's test in high dilution, the other with the colorless or lightly tinted fluid above described (Fig. 1). In the contents of the finest duct ramifications visible to the eye on section of the liver tissue, like differences were discernible. Several apparent exceptions in which a green bile was found where a colorless fluid had been expected served to emphasize the invariable nature of the rule.

On search, a communication with the gall bladder was discovered in every such instance, either by way of a fistula or through reestablishment of the old connection.

*Causes for the Differing Types.*

It is clearly evident how the contents of a green system, as we may call one connected with the gall bladder during stasis because of the characteristic hue, comes to be highly pigmented and at last tarry. In a companion paper we have demonstrated that the normal gall bladder effects a great and rapid concentration of the bile. One has only to suppose that the organ still functions in some measure during stasis to explain the heaping up of pigment in it and the connecting ducts. The change from brown to green is, for the most part at least, a simple oxidation of bilirubin to biliverdin.<sup>2</sup> The thickening to a heavy syrup and eventually to a tar or jelly occurs through the gradual accumulation of a mucinous nucleoprotein which is a normal product of the bladder mucosa.<sup>2</sup>

What, now, is the derivation of the colorless material distending a "white system," one obstructed out of connection with the gall bladder? There are several possibilities, but a discussion of them is unnecessary since our findings point in a single direction. The thin, colorless fluid is not bile at all but a product of the duct wall that has gradually replaced the small amount of hepatic secretion originally pent up.

*Experiment 4.*—A greater or less portion of the common duct with, in some instances, the trunks of the larger hepatic ducts was isolated in five dogs by ligating and cutting it above and below. After various periods up to 12 days the animals were chloroformed and examined. The isolated duct segment was found uninflamed but greatly distended in every case—up to a diameter of about 1 cm. on the average—and held several cubic centimeters of colorless and watery, or thinly mucinous, fluid, identical in its obvious characters with that of a white system of ducts as above described. All of the animals had become jaundiced as result of the total obstruction, yet none of the duct fluids was bile-stained and the two that were submitted to Hay's test gave a negative response. Cultures attested their sterility.

*Experiment 5.*—In a female dog of 11.5 kilos a segment of common duct was isolated and connected by means of a glass cannula and flexible rubber tube with a small, empty rubber balloon, which was left in the abdominal cavity when the abdominal wound was closed. Recovery from the operation was prompt, and the

animal remained in excellent condition during the 6 days prior to reexamination. The bag now held 8.5 cc. of a clear, sterile, watery fluid, slightly alkaline to litmus, and with a specific gravity of 1.011. The mucosa of the duct was not inflamed. A few degenerating epithelial cells separated from the fluid on standing, and when looked at in long column the latter had a faintly greenish cast, doubtless as result of changes in the bile pigment originally left in the duct. Hay's test for cholates was negative. The dog had not yet developed a tissue icterus.

These experiments demonstrate that the mucosa of the duct secretes a fluid of its own and is not prevented from so doing by a pressure sufficient to stretch considerably the tough and rather inelastic duct wall. The degree of distension observed was about equal to that in the closed ducts above the isolated segment. In this relation it is interesting to remember that the pressure within the ducts rises during stasis in the dog to equal that of a column of bile approximately 300 mm. high.<sup>6,7</sup> The amount of fluid produced in Experiment 5 in the absence of such a pressure obstacle was no negligible one—8.5 cc. in 6 days from a strip of mucosa about 2 cm. long and 0.7 to 0.8 cm. broad when the duct was laid open longitudinally.

#### *Histological Changes.*

The hepatic tissue in connection with obstructed ducts filled with colorless bile does not differ in the least in appearance, even after weeks of stasis, from that giving into a green system in the same animal. When the ducts from part of the liver have been left open there will be noted a general dilatation of the blocked channels, a slowly developing, orderly, interlobular cirrhosis, a few pigment thrombi between the parenchymal cords, and a more or less marked parenchymal atrophy in the region of stasis with compensatory hypertrophy elsewhere. When obstruction is total and jaundice has been present for some weeks one will find in addition marked parenchymal icterus and many intralobular bile thrombi, but still no differences referable to the green and white systems. Coursing beside the distended colorless ducts of the latter, and away from the liver, may be seen lymphatics turgid with

<sup>6</sup> Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

<sup>7</sup> Mitchell, W. T., Jr., and Stifel, R. E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 78.

fluid colored a bright yellow with bilirubin, and yielding a positive Hay's reaction. It is clear that the liver must be forming bile which is somehow prevented from entering the usual channels. The preventive agent can scarcely be other than the colorless secretion of the duct walls which has gradually backed up within them.

*Development of a White System.*

A systematic study has been made by us of the various steps in the development of a white system in the dog. During the first few days of obstruction the duct contents is still pigmented, appearing indeed somewhat more so than normal bile, owing to the conversion of part or all of its bilirubin into the much darker biliverdin. After about a week, as a rule, the pigment has become much less in amount, though in the lack of any criterion as to its original quantity, which varies greatly, of course, from animal to animal, examples cannot be given.

By the 10th or 11th day the duct contains a practically colorless fluid. In eleven dogs with total obstruction lasting from 8 to 27 days and icterus in varying degree, it was completely colorless, and devoid of cholates. So too in three cats with pronounced jaundice after 11 to 14 days of obstruction. Many instances could be cited of perfect white systems in animals with a partial obstruction, but these have less interest, owing to the fact that the occurrence of icterus was prevented in their case through a vicarious elimination of bile by the unobstructed liver portions. We have said that very few formed elements are present in the duct fluid. Cholesterol is practically absent as shown by the Liebermann-Burchard test which is occasionally negative and at most weakly positive. All these facts are as true for the contents of branches from single lobes, separately obstructed, as for that of the large channels.

Authorities are not agreed as to the precise point of escape of the bile from obstructed ducts but it is known to be close to the margin of the lobuli. According to Heidenhain<sup>8</sup> and most workers it is situated at the junction of the intralobular bile capillaries with the collecting channels of Glisson's capsule. Bürker<sup>9</sup> places it within the lobuli but

<sup>8</sup> Heidenhain, R., Studien des physiologischen Instituts zu Breslau, Leipsic, 1868, No. 4, 234.

<sup>9</sup> Bürker, K., *Arch. ges. Physiol.*, 1900-01, lxxxiii, 241.



near to their periphery. However this may be, one can suppose that in the gradual formation of a white system the fluid that accumulates in the ducts, diluting and replacing their original content, escapes at the same point as the bile. Certainly it must escape far back toward the lobuli, else the ducts in Glisson's capsule would be observed to contain bile, not colorless fluid when the liver is cut open. One may envision a slow upward current of duct secretion meeting and opposing a more rapid one downwards of bile in the region of the duct radicles, and the escape of both together through the walls.

In the monkey a "white bile" completely devoid of pigment and cholates is not obtainable, owing to the great distensibility of the ducts, so that relatively large amounts of stasis bile collect in them; to the short period during which they remain obstructed when cut between ligatures; and finally to one of several factors now to be discussed, which, acting likewise in the dog, often prevent the elaboration of a perfect "white bile." These are: (1) derivation of the white system from an hepatic lobe having another outlet which is patent; (2) the presence of gall bladder mucosa in the white system; (3) long continued obstruction.

In another connection<sup>10</sup> we have furnished evidence that the bile radicles which unite to form each primary hepatic duct fail in the dog to anastomose in any significant degree with those of other ducts. Each drains what may be termed a separate watershed, and when its outlet is blocked, as when the duct is tied, the tributary region suffers to its outermost limits, as is shown by the eventual sharp line of demarcation between normal liver parenchyma and that of the region in stasis, a line which follows closely the anatomical limit of the obstructed ducts. But physiologically the separation is not quite absolute, as the present work shows. Repeatedly in the course of it we have had opportunity to note the influence of a free duct on the contents of a white system, having its ramifications in the same tissue mass. Under such circumstances the fluid of the white system is regularly yellow with bilirubin and contains cholates, although other white systems deriving from entire lobes of the same liver have contents colorless and negative for bile salts. The fact that the pigment

<sup>10</sup> McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1921, xxxiii, 731.

in the affected white system is bilirubin, not biliverdin as in old stasis biles, argues its recent production. The most reasonable explanation of its presence would seem to be a constant slight secretion of bile into the ducts as result of a lowering of pressure in them consequent in turn upon a slight continual leakage from the white system over into the adjoining unobstructed region. How and where this leakage occurs cannot be said. In the monkey there is evidence for a considerable amount of it in the development regularly of a broad zone of transition between the liver tissue that is atrophic as the result of transmitted pressure from obstructed ducts and the adjoining normal parenchyma.

We have called attention in another paper to a difficulty encountered in attempts to block off completely the gall bladder of the dog without encroachment on the duct. The bladder mucosa often extends below the neck of the organ, to or beyond the entrance of the first channels from the liver, as is readily seen when the duct is laid open. The development of a new gall bladder after cholecystectomy is probably traceable to this arrangement, for it never occurs when the segment of duct in question immediately next the bladder has been ablated.<sup>11</sup> So great is the concentrating activity of the bladder upon the bile that when a ligature is laid on the neck of the organ the small residual portion left below may suffice, as we have shown, to effect a considerable reduction in the fluid bulk of the secretion.<sup>4</sup> It is to such a reduction in fluid bulk, whereby a little bile is enabled to enter a white system, that some of our anomalous instances should be attributed. In these cases when the duct distended with pale yellow bile was slit open a still more distended segment of characteristically thin walled bladder mucosa disclosed itself below the ligature which had been supposed to exclude it entirely. The neighboring ducts which had been separately obstructed held by contrast a colorless fluid.

Finally, in several dogs in which a white system and a general jaundice had existed for 6 weeks or more the stasis fluid was yellow and contained cholates in the absence of the factors just mentioned. The duct wall was now stretched very thin. Lessened secretion from it, or seepage through it, may have occurred.

<sup>11</sup> Haberer, H., and Clairmont, P., *Verhandl. deutsch. Ges. Chir.*, 1904, xxxiii, pt. 2, 81.

*The Changes in a Green System.*

The activities of the gall bladder during stasis merit special attention because they perhaps have no small share in the production of gall stones. The changes that occur within a white system are all in the direction of dilution and replacement of the bile. Those in a green system on the other hand seem at first view to be wholly the result of progressive biliary concentration. Within the first few days the bladder contents turns greenish black and as weeks elapse it thickens to a tar or jelly, while similar though less marked alterations occur in the fluid of the connecting ducts. It has required quantitative observations to show that here is no gradual accumulation of pigment; and in a preliminary note we have expressed this erroneous view.<sup>12</sup> What really happens is a progressive conversion of bilirubin to the greatly darker biliverdin, whereby a gradual disappearance of much of the pigment is masked. The point can be readily demonstrated by diluting out the bladder bile with water. The bile obtained after a week or 10 days of obstruction, though colored a green-black, dilutes out through the yellow-brown of bilirubin, to a bright yellow which only disappears in relatively large quantities of water. After another week the green persists to a higher dilution, but the proportion of water required to bring about a total disappearance of color is not so great. Finally after 5 weeks or more in the dog, and much sooner in the monkey, the yellow tint is missing when water is added to the bile and only a clear green is got which fails to survive much dilution. The disappearance of pigment thus indicated is also shown by titrations carried out according to Hooper and Whipple's method. At the latest period of stasis that we have studied—44 days, in the dog—the bladder contents still reacts characteristically with acid alcohol, and colorimetric readings against a bilirubin standard can be made. The amount of pigment found per cubic centimeter is now not more than twice that of many normal biles, instead of six to ten times the quantity as during the first 10 days.

How soon after the production of a green system does the concentrating activity of the gall bladder cease, and what are the maximum alterations effected by it? These questions are not readily answered.

<sup>12</sup> Rous, P., and McMaster, P. D., *Proc. Soc. Exp. Biol. and Med.*, 1920, xvii, 143.

TABLE I.  
*Character of Stasis Bile from the Gall Bladder.*

Dog No.	Dura- tion of obstruc- tion.	Contributing liver lobes.	Liver per cent.	Jaundice.	Character of stasis fluid.	Diluting to.	Pigment per cc.	Sodium tauro- cholate per cc.	Hay's test + at dilution of.	Remarks.
	<i>days</i>						<i>mg.</i>	<i>mg.</i>		
1	2	Right and left central; pa- pillary.	43	0	Dark brown, syrupy.	Yellow.	2.7	32.2	1 in 80	Other lobes unob- structed.
2	4	All except cau- date and pa- pillary.	83	Slight.	Brown-black, syrupy.	"	5.9	35.0	1 " 64	Other lobes unob- structed. No tissue jaundice but bilirubin in blood and urine.
3	9	Caudate, right lateral; half of right central.	42	0	Thick olive- black, syrupy.	"	2.8	36.7	1 " 64	Other lobes unob- structed.
4	9	All except cau- date.	88	0	Thick olive- black, syrupy.	Greenish yellow.	4.3	34.0	1 " 40	Other lobes unob- structed.
5	9	Right central; papillary.	32	+	Viscid, green- black.	"	3.2		1 " 20	Other lobes ob- structed.
6	9	Main liver mass.	73	+	Viscid, green- black.	"	5.7		1 " 40	Other lobes ob- structed.
7	10	Right and left central; pa- pillary.	43	0	Thick greenish black syrup.	"	3.8	10.2	1 " 64	All other lobes ex- cept caudate ob- structed.
8	12	Right and left central.	38	+	Thick greenish black syrup.	Yellow-green.	3.0		1 " 32	Other lobes ob- structed.

9	34	Right central and papillary.	31	0	Thick green-black syrup.	Green.	1.9	63.1	1 in 192	Caudate and right lateral also obstructed.
10	36	Right half of right central.	12	0	Tarry, dark green.	Yellow.	1.0	16.4	1 " 128	Other lobes save caudate and right lateral are also obstructed.
11	36	Right central and papillary.	26	0	Tarry, dark green.	Green.	0.9	56.6	1 " 64	Other lobes excepting right lateral and a part of caudate are obstructed.
12	44	Main liver mass and papillary.	65	0	Tarry, green-black.	Yellowish green.	1.2	8.5	1 " 256	Part of caudate lobe also obstructed.

The inspissatory changes come about entirely through osmosis and diffusion, and are limited by the  $\Delta$  of the blood.<sup>13</sup> After the first few days the bladder and ducts undergo no further dilatation, their capacity when they are taken together being now only twice to four times the normal. It follows that the stasis bile will be mostly a derivative of that purveyed by the liver during the first few days of obstruction. And since the constitution of ordinary bile in substances of large molecule varies much, it follows that the small space available for its accumulation may in some individuals come to be occupied largely by substances of one sort to which the bladder wall is impermeable, and again by those of another. This fact is reflected in the irregularities of Table I.

In none of the fluids recovered after 2 days or more of stasis was so great a concentration of pigment found as in some bladder biles acted upon by the normal organ for 24 hours.<sup>4</sup> After 9 days of stasis the pigment is still unincreased, and later there is a gradual diminution in it. One may conclude that the maximum pigment concentration is effected during the first day or two of obstruction, and that thereafter the activity of the gall bladder in this regard practically ceases.

Quantitative observations on cholesterol were not made because the bladder mucosa itself is recognized to be a source of the substance. Cholates though were estimated in two ways, as already stated,—by Foster and Hooper's amino-acid method and by Hay's sulfur test carried out on progressive dilutions of the bile.

Both of these methods as used in connection with stasis bile are open to criticism. An inspissated bile long kept at body temperature may very well come to contain other substances besides salts of taurocholic acid that fail to come down in boiling alcohol and will yield amino-acids on hydrolysis. Derivatives of the abundant nucleoprotein in special might well do this. Hay's sulfur test is not only given positively by any substance that lessens surface tension, but the lessening ordinarily caused by cholates may be hindered by other bile constituents. Thus, for example, we have several times found that a watery solution of sodium taurocholate which yielded a positive Hay's test when mixed with a given amount of water, failed to do so in the presence of a very slight trace of bile although the latter itself contained cholate but not in quantity sufficient to elicit a reaction in the dilution employed. There are then several possible explanations for the differences in result of the two methods (Table I).

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<sup>13</sup> Brand, J., *Arch. ges. Physiol.*, 1902, xc, 491.

The general indications obtained by both methods are that bile salts, unlike pigment, may increase in concentration as stasis progresses.

The gradual viscid thickening is the result of the persistent elaboration by the bladder wall of a mucinous material identified by Hammarsten as a nucleoprotein.<sup>2</sup> This is not produced in any quantity by the ducts. At first only the contents of the receptaculum chyli are thickened with it, but later it extends into the larger and smaller passages, and finally the most minute ones visible to the unaided eye are distended with tiny plugs of green jelly. In this connection it is noteworthy that the distribution of pigment and cholates throughout the green system eventually becomes an approximately even one. At first these are principally massed in the gall bladder, as would follow from the localization of the concentrating activity to this latter.

*Experiment 6.*—In thirteen dogs, one cat, and one monkey, with green stasis systems of 2 to 36 days duration a clamp was placed upon one of the tributary ducts at autopsy and the fluid obtained from above was compared with the bladder contents as regards pigment and cholates. So little material was yielded by the duct that Hay's test of graduated watery dilutions, and a direct color comparison with similar dilutions of the bladder contents, were the best methods available. They regularly indicated at first marked differences between the two biles, which tended to disappear later. Thus after 4, 9, and 10 days of obstruction, respectively, in three dogs the pigment was only 54, 25, and 30 per cent as strong in the duct fluid as in the gall bladder, whereas in three other dogs kept for 34, 36, and 36 days, its relative strength was 80, 86, and 100 per cent.

More factors influence the changes in a green stasis system than in a white one. The differing permeability of the bladder wall for different substances, and the eventual clogging of the mucosa which will vary in its effects with the substance; the continued production of mucus; the activity of the ducts to dilute the bile; these and doubtless other moments interact to alter the stasis fluid. The eventual tendency is toward replacement of the bile with duct secretion and mucinous material from the bladder.

*The Pressure in Green and White Systems.*

The fluid in obstructed bile ducts is pent under a day-to-day pressure averaging about 300 mm. of bile in the dog,<sup>7</sup> a sufficient evidence that there are considerable barriers to its escape. These barriers would appear to be equally effective for the green and white systems. The distension of the ducts is the same in both when present in a single liver, and, what is perhaps more significant, the hepatic regions pertaining to the two show no noteworthy differences. We have pointed out in a previous paper<sup>10</sup> that the pressure in regions of stasis has effect in a diversion of the portal stream with local parenchymal atrophy and a compensatory hypertrophy elsewhere. The changes may be readily followed in the dog liver, where they are marked after but a few weeks. Now were the duct pressure in a green or a white system much greater than in its fellow there should be a reflection of this in differing degrees of parenchymal change. Such are not found. The activity of the gall bladder, while sufficient to determine the character of the stasis bile, is, then, without enduring influence on the stasis pressure. The fact that the pressure developing when ducts are suddenly obstructed is the same as that on their continued stasis<sup>4,7</sup> suggests that the prime moment in its maintenance is the secretory activity of the liver.

*Some Results of Infection.*

Consideration has been given thus far only to happenings in the absence of infection. The entrance of this latter may bring with it innumerable complexities. Two that have come under our eye will be put on record, since they throw light upon the problem of obstruction in man.

The fluid elaborated by infected ducts is sometimes no longer thin but so viscid as practically to occlude the ducts. In a dog from which we were making day-to-day collections of the bile from different portions of the liver, infection occurred of one of the duct systems drained by rubber tubes. Almost at once its output became thickly mucinous and practically lost the biliary character, being but faintly tinted with bilirubin whereas the companion bile that served as control was still thin and dark. At autopsy the liver



proved to be normal; and portions of the control bile when incubated with organisms from the infected ducts failed to thicken. There is little doubt that here we had to do with cholangitis rendered obstructive by a thick secretion from the duct walls; that is to say, just such a condition of affairs as is supposed to be operative in the catarrhal jaundice of human beings.

Infection may so change the gall bladder that a white system develops where a green is expected. In one of our dogs chronic infection led to a thickening of the bladder wall, and this organ and all of the obstructed ducts connected with it were found distended with a colorless, syrupy fluid that failed to give Hay's reaction but contained small ropes of old pus and numerous micrococci. There is good reason to suppose that in this instance the infection prevented an inspissation of bile by the bladder.

#### DISCUSSION.

It has long been taken for granted that the walls of the ducts influence the bile as do those of the gall bladder, and this view has colored the interpretation of pathological findings. That it is incorrect the present observations show. The activities of ducts and bladder are opposite in nature, but of such different magnitude that those of the bladder determine the picture when the bile is submitted to both. It is a curious fact that the duct walls, though intimately related to the tissue forming the bile, themselves secrete a fluid that is colorless even when the animal is heavily jaundiced. The glands of the wall behave in this respect like those forming the tears and saliva and those of the gastric mucous membrane,<sup>14</sup> as contrasted with the sweat glands and kidneys which put out bilirubin during icterus. It is true that the fluid in a white system with walls stretched thin after long standing total obstruction may be lightly tinted with bilirubin and contain cholates; but these substances not improbably come from the liver under such circumstances. The fact may be recalled in this relation that mucous surfaces in general become permeable to bile pigment when inflamed.<sup>14</sup>

<sup>14</sup> Krehl, L., *Pathologische Physiologie*, Leipsic, 9th edition, 1918.

"White biles" have been obtained from human beings with extreme fatty degeneration of the liver<sup>15</sup> as well as after duct obstruction. The instances on record leave little doubt that a liver with injured parenchyma sometimes yields a colorless fluid having scarcely any resemblance to true bile. Our present findings warrant the suggestion that the fluid is derived from the duct walls, while the liver cells proper have practically ceased secreting. However this may be, there is no doubt that the ducts are the source of "white bile" in obstructive instances. In them the integrity of the parenchyma is attested by the resumption of secretion into the old channels when the impediment has been removed.<sup>16</sup> The condition of affairs while the obstruction holds is essentially similar to that studied by Heidenhain<sup>8</sup> in his classic experiments on the introduction under pressure of sodium indigotate into the ducts. The strange fluid, whether indigotate or white bile, fills all the extralobular passages, preventing any bile from entering them, and its surplus escapes with the bile itself through the walls of the duct radicles at or near the margins of the liver lobuli. When pressure is relieved the bile once more takes its way into the proper channels, flushing out ahead of it the strange fluid. Secretion by the liver is never inhibited, only diverted, a fact abundantly proven by the jaundice that ensues when the obstruction has been total.

In many instances in man of white bile from obstructive causes the hepatic duct or a branch of it has alone been blocked, but in others the common duct is occluded and the gall bladder and all the passages fill with a colorless fluid.<sup>16</sup> Here is an apparent contradiction to our rule on the origin of green and white systems. But a green system can only be produced when the gall bladder is capable of concentrating bile during stasis. Lacking this ability the organ becomes a mere diverticulum in a white system. And it is with such incapable gall bladders that one has to do in these anomalous instances. Aschoff and Bacmeister<sup>17</sup> have emphasized the fact in

<sup>15</sup> Ritter, E., *Compt. rend. Acad.*, 1872, lxxiv, 813. Robin, A., *Compt. rend. Soc. biol.*, 1884, xxxvi, 115.

<sup>16</sup> Kausch, W., *Mitt. Grenzgeb. Med. u. Chir.*, 1911, xxiii, 138. Fischler, F., *Physiologie und Pathologie der Leber, nach ihrem heutigen Stande mit einem Anhang über das Urobilin*, Berlin, 1916.

<sup>17</sup> Aschoff, L., and Bacmeister, A., *Die Cholelithiasis*, Jena, 1909.

connection with hydrops that the normal gall bladder does not distend with fluid when tied off at the neck but draws gradually down into a small, thick walled globe containing a little mucous jelly. This happens even during the jaundice of total obstruction, as we have had occasion to note. Hydrops, as Aschoff and Bacmeister rightly say, is the expression of a change in the bladder wall such that fluid is elaborated by it instead of withdrawn through it. When an organ pathologically active in this way, or one merely indifferent to the bile, stands in connection with an obstructed duct system there will inevitably occur a gradual replacement of the original stasis bile with a secretion derived from the duct walls. We have furnished a specific instance in the animal already mentioned that was operated upon for the production of a green system, but in which a white developed instead, in connection with a bladder thick walled from chronic infection. Kölliker and Müller<sup>18</sup> noted as far back as 1856 in a dog with an ill cared for gall bladder fistula that a glairy, colorless fluid collected when the fistulous opening closed for a day or two, which was replaced by bile after some hours of drainage.

A recognition of the physiological influences which make for the production of green and white stasis systems should bring some order into the chaos of observations on stasis bile. One is enabled to say with certainty that here the concentrating activity of the gall bladder is mainly responsible for the character of the fluid found, and that there it is the product of the ducts and in certain instances of an hydropic bladder. But the diverse infections to which the biliary passages are liable bring with them innumerable complexities. One we have recorded in the sudden mucinous thickening of duct bile, with obstruction as a result.

The differing activities of the bladder and ducts bear directly upon the problem of cholelithiasis. Our observations leave little ground for surprise over the fact that stones of the hepatic duct or its branches are relatively infrequent and give but little trouble clinically. A greater or less degree of obstruction of these channels must often occur: it is inevitable to inflammatory or neoplastic changes in the liver tissue. But such local stasis as may thus be caused is followed,

<sup>18</sup> Kölliker, A., and Müller, H., *Verhandl. physik.-med. Ges. Würzburg*, 1856, vi, 435.

as the present experiments show, not by a concentration of the stagnating bile, but by its dilution and replacement with a fluid from which one of the principal substances forming stones, bilirubin, is often completely absent and the other, cholesterol, sometimes practically so. The fluid is thin, like the liver bile itself, and both will readily find a way around stones that only partially occlude a duct.

The participation of the gall bladder completely changes the conditions and the ultimate prospect. We have shown how rapidly the organ acts to inspissate the bile, even in the absence of stasis.<sup>4</sup> Aschoff and Bacmeister bring evidence that most stones have their beginning in an uninfected and approximately normal gall bladder. In their view cholesterol falls out of the incubated bile in crystalline form and deposition takes place thereon; while Naunyn<sup>19</sup> and many others believe that bacteria and cellular debris constitute the nuclei of formation. Whatever the true case, this much at least is certain, that the enlargement of stones is by a deposition out of solution or suspension. The activity of the gall bladder to concentrate the bile cannot but be of profound importance in this connection. Indeed, if one accept the cholesterol hypothesis of stone origin it assumes primary significance. The very rapid "growth" of stones that have passed into the common duct and cause partial obstruction, as described by Naunyn, and the secondary formation in such cases of stones just above, are alike attributable in large part to the influence of the bladder upon repeated fresh increments of bile. And the danger of total obstruction under such circumstances is rendered greater by the thickening of the bile with bladder mucus.

We have already pointed out that the upper portion of the cystic duct often has the physical characters of the bladder wall and with them its concentrating ability.<sup>4</sup> This latter fact will go far to explain the development of stones in the duct after cholecystectomy, an occurrence not infrequent in the days before duct ablation was practised.

Aschoff has an aphorism to the effect that the essential and common cause of all gall stones is biliary stasis. One might say for many

<sup>19</sup> Naunyn, B., A treatise on cholelithiasis, Sydenham Society translation, London, 1896.

cases, as coming nearer to the actual event, that it is biliary inspissation.

Stones rarely develop as a result of continuous obstruction. Under such circumstances, as we have shown, bile soon ceases to come from the liver into the closed passages, and that which at first assembles becomes thickened with mucus from the bladder and gradually diluted with the secretion of the duct walls. In none of our old bladder biles was any particulate matter found except desquamated cells.

How do these facts bear on the present vogue of cholecystectomy? It is evident from them that the concentrating activity of the gall bladder renders it a menace during intermittent stasis and whenever the bile itself is of such kind that stones readily form out of it. The more normal the organ, or to speak precisely, the more of the concentrating faculty it retains, the greater is the danger. On the other hand, the surgeon should realize that the removal of a normal gall bladder entails, as we have pointed out in a companion paper,<sup>4</sup> functional disturbances that are none the less significant because the body adjusts itself to them. Furthermore, as Oddi originally showed, cholecystectomy is often followed by a marked and permanent dilatation of all the ducts so that they come to hold much bile. The accumulated secretion is separated from the intestine only by a weakened sphincter, and ascending infection of the ducts has been noted to occur.

In patients with a tendency to stone formation it would seem wise to prevent, so far as possible, concentration of bile by the gall bladder. A simple expedient suggests itself to this end, namely frequent feeding. During fasting periods not only is bile stored in quantity and concentrated by the bladder but the secretion as it comes from the liver is rich in solids.<sup>20</sup> The passage of chyme through the duodenum is accompanied by an expulsion of the bladder contents<sup>21</sup>

<sup>20</sup> Stadelmann, E., *Der Icterus und seiner verschiedenen Formen*, Stuttgart, 1891.

<sup>21</sup> Bruno, G. G., *Dissertation*, St. Petersburg, 1898, and Klodnizki, *Dissertation*, St. Petersburg, 1898; cited by Babkin, B. P., *Die äussere Sekretion der Verdauungsdrüsen*, Berlin, 1914, 344. Bruno, G. G., *Arch. sc. biol. St. Pétersbourg*, 1899, vii, 87.

and its replacement with an abundant, thin flow from the liver. Viewed as prophylaxis, the oftener this can happen the better.

#### SUMMARY.

The gall bladder and ducts exert opposite influences upon the bile. The ducts fail to concentrate and thicken it with mucus as the bladder does, but dilute it slightly with a thin secretion of their own that is colorless and devoid of cholates even when the organism is heavily jaundiced. The fluid may readily be collected into a rubber bag connected with an isolated duct segment. It continues to be formed against a considerable pressure, and, in the dog, is slightly alkaline to litmus, clear, almost watery, practically devoid of cholesterol, and of low specific gravity to judge from the one specimen tested. In obstructed ducts separated from the gall bladder, or connecting with one so changed pathologically that the concentrating faculty has been lost, such fluid gradually replaces the small amount of bile originally pent up. It is the so called "white bile" of surgeons.

When obstructed ducts connect with an approximately normal gall bladder the stasis fluid is entirely different, owing to the bladder activity. At first there accumulates in quantity a true bile much inspissated by loss of fluid through the bladder wall, darkened by a change in the pigment, and progressively thickened with bladder mucus. As time passes duct secretion mingles with the tarry accumulation and very gradually replaces it. The inspissation of the bile, as indicated by the pigment content, is at its greatest after only a day or two of stasis.

The differing influences of the ducts and bladder upon the bile must obviously have much to do with the site of origin of calculi and their clinical consequences. The concentrating activity of the bladder cannot but be a potent element in the formation of stones. We have discussed these matters at some length. Intermittent biliary stasis is admittedly the principal predisposing cause of cholelithiasis; and the stasis is to be thought of as effective, in many instances at least, through the excessive biliary inspissation for which it gives opportunity. In this way a normal gall bladder can become,

merely through functional activity, a menace to the organism. In patients with the tendency to stones frequent feedings may lessen the danger of their formation.

#### EXPLANATION OF PLATE 4.

FIG. 1. White and green stasis systems after 11 days of total obstruction with jaundice. Retouched photograph. The gall bladder and connecting ducts, *A, A, A*, are dark with heavily pigmented green bile, while the branches, *B, B*, of a separately ligated duct distended with "white bile" are translucent and practically colorless.

FIG. 2. "White bile" and green from the same animals. The contrasting specimens, *A* and *B*, were obtained after 22 days of obstruction in a dog with one large duct left open, and consequently no icterus; and *A'* and *B'*, after 26 days of total obstruction with icterus.







FIG. 1.

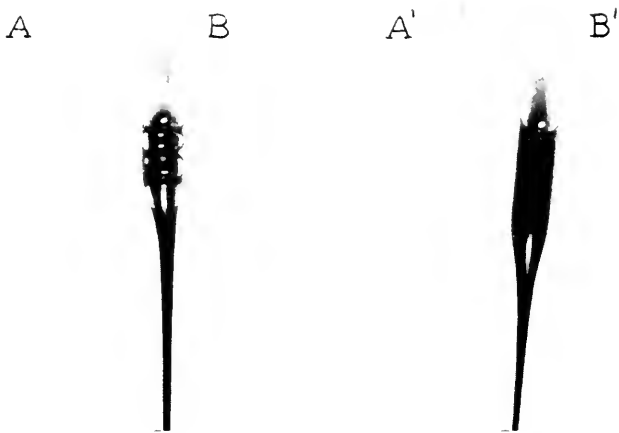


FIG. 2.

(Rous and McMaster: Stasis bile.)



## STUDIES ON BACTERIAL NUTRITION.

### II. GROWTH ACCESSORY SUBSTANCES IN THE CULTIVATION OF HEMOPHILIC BACILLI.

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In a preceding paper it was shown by Thjötta (1) that *B. influenzae* will grow on hemoglobin-free medium consisting of plain broth enriched by sterile suspensions or extracts of mucoid bacteria. It was suggested that under these conditions growth may be attributable in part to the presence in the bacterial extracts of substances belonging to the class of so called vitamins. It was found that the growth-promoting action of these substances of bacterial origin was not destroyed by boiling for 10 minutes or by passage through Berkefeld filters. In the present paper are presented the facts thus far acquired in the study of the growth requirements of the so called hemophilic bacilli: the growth-stimulating action of extracts of yeast and of vegetable cells, and the importance of blood as a source of growth accessory substances in the cultivation of bacteria.

The earliest reference in literature to the importance of accessory growth factors in the cultivation of microorganisms is made by Wildiers (2) (1901). This investigator observed that watery extracts of yeast cells greatly enhance the growth of yeast in a synthetic medium.

In confirming the observation of Pasteur on the growth of yeast in a medium composed of mineral salts and sugar, Wildiers found that the size of the inoculum was of the greatest importance; a small seeding would not suffice to initiate growth, while a larger inoculum grew abundantly in this medium. This fact suggested that the growth difference lay in some intracellular factor furnished by the heavier seeding which on the death and disintegration of certain of the yeast cells was liberated, and supplied to the surviving cells a necessary growth-inducing substance. This growth-stimulating principle in yeast to which Wildiers gave the name "bios" was contained within the cell and could be extracted by boiling yeast cells in water. The extract obtained in this manner and concentrated on a water bath was a yellowish, clear, syrup-like fluid. This intracellular substance, the so called "bios," was soluble in water and 80 per cent alcohol, and insoluble in concentrated alcohol and ether. It was resistant to acids but sensitive to alkalis. It could be filtered and dialyzed through paraffined paper.

The findings of Wildiers concerning "bios" were confirmed by Amand (3) who showed that this substance disappeared from the culture medium during growth. This observation Amand interpreted as evidence that "bios" was used up by the yeast cells during growth, and was not a product of their own metabolism.

The work of Wildiers and Amand seems to have been forgotten until quite recently when several investigators have adapted the principle of these earlier workers to methods for the measurement of the vitamine content of various substances. Bachmann (4) measures the vitamins in the test material by adding the sterilized substance to yeast cultures and measuring the amount of gas evolved as an index of growth acceleration; Eddy and Stevenson (5), in determining the presence of vitamine, compare the numerical increase of cells in cultures of yeast in a medium with and without the test substance. On the other hand, from their experiments on the growth of yeast in synthetic media without growth accessory substances, McDonald and McCollum (6) conclude that yeast must be capable either of growing without vitamins or of synthesizing these substances during growth.

While Wildiers and Amand distinctly pointed out the importance of extracts of yeast in the nutrition and growth of yeast cells, they did not extend their observations to the effect of these extracts upon the growth of other microorganisms, such as bacteria. It was not until after the discovery of the value of growth accessory substances in the nutrition of animals that the question of their significance in the growth of bacteria was considered. Bottomley (7) showed the existence in peat of certain substances that have a stimulating effect upon the growth of plants and soil bacteria. These substances he called "auximones" and considered them of the same importance in the growth of plants as vitamins in the nutrition of animals. These "auximones" can be extracted from peat in the same manner as the vitamins from plants, and this fact, together with their apparently uniform action seems to indicate the close relationship between these substances.

Following this discovery it was only a question of time when the principle of growth accessory substances should be brought into full use in the cultivation of bacteria.

In 1916, Lloyd (8) showed that the meningococcus required for its growth a rich supply of vitamins in the medium. Lloyd attributes the value of blood and serous fluids in the cultivation of meningococcus to their vitamine content and believes that Gordon's pea flour and Vedder's starch medium may also contain vitamine which may partly account for the value of these media. In explanation of the fact that meningococcus after artificial cultivation tends to grow with increasing readiness on ordinary media, this author suggests that in adaptation to a more or less saprophytic existence these organisms become independent of vitamine supply.

Cole and Lloyd (9) recommend as the optimum medium for gonococci one rich in amino-acids and vitamins, or "growth hormones." These authors

believe in the existence of two different "growth hormones," a substance present in blood cells and easily absorbed from the media by filtering, which seems to induce the initial growth, and a second substance found in tissues of plants and animals which has the power of inducing luxuriant secondary growth. Davis (10) pointed out that *B. influenzae* requires in addition to hemoglobin a vitamine substance. He compared these two factors to the fat-soluble vitamine A and the water-soluble vitamine B. Davis showed that the vitamine supply could be obtained from plant and animal tissue. By placing sterile sections of vegetables on blood agar seeded with influenza bacilli, Davis demonstrated that better growth occurred around the pieces of vegetables than on the other portion of the plates, just as Grassberger many years ago called attention to the more luxuriant growth of Pfeiffer's bacillus around colonies of staphylococci.

Davis (10) also applied this principle in the cultivation of other bacteria. As sources of vitamins Davis added to media polished and unpolished rice flour, white and whole wheat flour. If these grains were allowed to sprout the growth was more profuse. The increasing growth on the media made from sprouting grain is explained as due to enzymotic activity in the sprouting process, resulting in changes in proteins, and inversion of starch into sugars.

In the preceding paper (1) on the growth of *Bacillus influenzae* in broth containing sterile bacterial extracts, it was pointed out that this phenomenon may be explained in part at least by the presence of growth accessory factors, or vitamins, in the bacterial extracts. In this paper an attempt is made to analyze further the growth requirements of the so called hemophilic bacilli and to indicate the significance of growth accessory substances in bacterial nutrition.

#### EXPERIMENTAL.

##### *Growth Accessory Substances in Yeast and Vegetable Cells.*

Although extracts from bacteria of the mucoid variety may be readily prepared, their use is not practicable. In the present study extracts were prepared from yeast cells which are known to be rich in water-soluble vitamins, and which, as previously shown by Wildiers and Amand, have a marked stimulating effect upon the growth of yeast cultures. Extracts of fresh ripe tomatoes, green peas, and green beans were also used, since these vegetables are also valuable sources of growth accessory substances.

*Preparation of Extract of Yeast.*—100 gm. of brewers' yeast<sup>1</sup> were emulsified in 400 cc. of distilled water. Since the vitamins will stand boiling better in acid than in alkaline solution, the reaction of the suspension of yeast cells was adjusted to pH 4.6, boiled over a free flame for 10 minutes, and then allowed to sediment at room temperature. The clear supernatant extract was pipetted off and tested for sterility. The clear, sterile extract, unneutralized, was stored in the ice box and added to the medium immediately before use. The extract prepared in this manner was a clear, yellowish fluid. Chemical analysis showed the following nitrogen content:<sup>2</sup> total nitrogen, 0.116 per cent; ammonia nitrogen, 0.011 per cent; amino nitrogen, 0.039 per cent; peptide-bound nitrogen, 0.024 per cent; undetermined nitrogen, 0.042 per cent. Before addition of the yeast extract to media, the reaction may be readjusted to optimum for growth of *Bacillus influenzae*, pH 7.3 to 7.5.

*Preparation of Extract of Tomatoes.*—Ripe tomatoes were treated in the following way: The skin surface was seared with a red hot knife and through this area a sterile fork was plunged. The tomato was then dipped in alcohol and flamed, then plunged into boiling water for a minute, the skin peeled off, and the stem removed with sterile forceps. The tomatoes were placed in a sterile enamel dish, and crushed with a sterile pestle. The reaction of the tomato juice in its natural condition was pH 4.2, and therefore required no readjustment before boiling, as in the case of the yeast emulsion. The crushed tomatoes were boiled for 10 minutes and the expressed juice either filtered through a Berkefeld filter (N) or cleared by centrifugation, stored at its original acidity, and the reaction readjusted before use. This extract was a perfectly clear, slightly yellowish fluid. Nitrogen partition on this particular extract was as follows: total nitrogen, 0.14 per cent; ammonia nitrogen, 0.014 per cent; amino nitrogen, 0.079 per cent; peptide-bound nitrogen, 0.00 per cent; undetermined nitrogen, 0.047 per cent.

<sup>1</sup> The yeast used in these experiments was supplied through the courtesy of Mr. F. Spitzner of the Central Brewing Company of New York.

<sup>2</sup> For the nitrogen determination on both the yeast and tomato extracts we are indebted to Miss Alma Hiller of the Hospital of The Rockefeller Institute.

*Preparation of Extracts of Green Peas and Beans.*—Fresh green peas were prepared by flaming the surface and opening the pods with sterile forceps and crushing the separate seeds out into a sterile dish. An equal amount of sterile distilled water by weight was added and the reaction of the emulsion adjusted to pH 4.6. After boiling for 10 minutes the extract was strained through glass wool and then filtered through a Berkefeld filter (N). The resulting extract was perfectly clear and yellowish in color. Similar extracts were prepared from string beans.

*Stimulating Action of Vitamine-Like Substances on the Growth of Bacillus influenzae.*—Yeast and vegetable extracts prepared in the

TABLE I.

*Stimulating Action of Growth Accessory Substances in Yeast and Vegetable Extracts on the Growth of B. influenzae.*

Dilution of extract in plain broth.*	Growth-stimulating action of.		
	Yeast extract.	Tomato extract.	Extract of peas.
1:10	++	++	++
1:100	++	++	++
1:1,000	+	—	—
1:10,000	—	—	—
Plain broth.	—	—	—

\* The extract-containing broth was inoculated with 0.05 cc. of the supernatant fluid of a blood broth culture of *B. influenzae*.

++ indicates good growth; + moderate growth; ± slight growth; — no growth.

manner described were tested for their stimulating action on bacterial growth by adding them in varying concentration to plain broth of pH 7.8. This medium was inoculated with 0.05 to 0.1 cc. of the supernatant fluid of a blood broth culture of *Bacillus influenzae*. A bacterial whirl was often visible in the cultures seeded in this manner after 6 hours incubation, and was always marked after growth over night.

Titration of the vegetable extracts were made in infusion broth to determine the lower limit of the growth-stimulating action (Table I). Table I shows that even in high dilutions extracts of yeast and

of fresh green vegetables are able to stimulate growth of *Bacillus influenzae* in plain broth if seeded with a small inoculum from media containing blood. When it is considered that the original extracts contain little nitrogenous matter (yeast extract 0.14 per cent) and that only one-thousandth of this amount is present in the dilution required for growth, it becomes obvious that the extracts do not serve merely as additional nutriment, but that their action is accessory in nature, similar perhaps to that of vitamins in animal nutrition. This resemblance is the more striking in that these extracts resist boiling for at least 10 minutes and are destroyed at autoclave temperatures (120°C. for 30 minutes) as shown in Table II.

TABLE II.

*Relative Growth Capacity of Yeast and Tomato Extracts after Boiling and Autoclaving.*

Temperature.	Tomato extract.	Yeast extract.	No extract.
	In plain broth, 1:10.*	In plain broth, 1:10.*	Plain broth.
10 min. at 100°C.	++	++	—
30 " " 120° "	—	—	—

\* Inoculated with 0.1 cc. of the supernatant fluid from an 18 hour blood broth culture of *B. influenzae*.

In the literature the statement is frequently encountered that the growth value of culture media is greatly impaired by filtration. This loss is attributed to absorption of the so called hormones during the process of filtering. In order to test the effect on the growth-stimulating value of yeast extract after absorption with bone charcoal, the following experiment was carried out.

In each of two small flasks under sterile precautions 1 gm. of bone charcoal and 5 cc. of yeast extract (pH 5.4) were mixed. One portion of the mixture was heated on a steam bath for 15 minutes in order to facilitate absorption; the other was placed at room temperature and frequently shaken. At the end of the absorption period the charcoal was removed from suspension by centrifugation and the absorbed extract tested for its growth-inducing action. Extract without charcoal was heated on a steam bath for 15 minutes to determine whether or not the additional heating affected its potency. The results are recorded in Table III.



From Table III it is apparent that the growth-promoting power of yeast extract is susceptible to absorption by bone charcoal. It is, of course, obvious that the completeness of absorption is related to the concentration of the particular extract and the length of time allowed for absorption. It is evident that under the experimental conditions described the vitamine-like principle in yeast extract is absorbed by bone charcoal and that absorption occurs more promptly under the influence of heat.

From the data presented in the preceding experiments it is evident that *Bacillus influenzae* will grow when transferred by small inoculum from blood media to plain broth containing extracts of yeast or vege-

TABLE III.

*Effect of Absorption by Bone Charcoal on the Growth-Stimulating Action of Yeast Extract.*

Dilution of extract in plain broth.*	Yeast extract absorbed.		Unabsorbed.	
	15 min. on steam bath.	2 hrs. at room temperature.	Unheated.	15 min. on steam bath.
1:10	±	+	++	++
1:20	—	±	++	++
1:50	--	—	++	+

\* Inoculated with 0.05 cc. of the supernatant fluid of a blood broth culture of *B. influenzae*.

table cells but fails to grow under similar conditions in the same broth without the addition of these extracts. However, for reasons to be discussed later, continued cultivation fails in broth containing only yeast or vegetable extracts.

While the chemical nature of these growth accessory substances is not known, they are analogous in behavior to the so called vitamins. Extracts containing these substances have been prepared from fresh vegetables and from bacterial and yeast cells. It has been found that they resist boiling for 10 minutes, that they are destroyed by autoclaving, that they contain but little available nitrogen, that they pass a Berkefeld filter with little or no impairment, but are absorbed from water solution by bone charcoal.

*Growth Accessory Substances in Blood.*

Blood has always been considered requisite for growth of Pfeiffer's bacillus, and the inability of certain organisms to multiply in the absence of blood or blood derivatives has constituted an absolute criterion for their differentiation as hemophilic bacteria. It is important at this point to emphasize again the fact previously mentioned that although *Bacillus influenzae* grows luxuriantly when transplanted from blood medium to plain broth containing yeast extracts, cultivation cannot be continued for more than one or two transfers in yeast broth alone. Growth deficiency under these circumstances suggests that possibly some other substance may be carried over from the original blood culture in an amount sufficient to supplement the vitamine factor in yeast broth and that growth fails in succeeding cultures in this medium because this second substance is either exhausted by growth or lost by dilution on subsequent transfer. For purposes of discussion this second substance may be referred to as the "X" factor, and the vitamine-like substance as the "V" factor. The theoretical consideration of the presence of these two essential growth factors in blood requires for its substantiation the demonstration of the dual nature of the growth-stimulating property of blood. In the following experiments the interdependence of these two substances and their distribution in the various fractions of blood, body fluids, and crystalline hemoglobin will be discussed.

It has already been pointed out in the first part of this paper that one of the substances, the V factor, can be supplied from a source other than blood, as for instance, yeast. In explanation, therefore, of failure of continued growth of *Bacillus influenzae* in yeast broth alone, it is suggested that the X substance lacking in this medium is supplied in the first instance by the inoculum from the original blood broth, and that the amount of X furnished in this way is sufficient when supplemented by an excess of the V factor from yeast to sustain growth in the first transfer, but that in subsequent cultures the X factor is quickly lost or perhaps used up by growth of the bacilli. That this second substance, the so called X factor, is actually carried over from blood broth with the first inoculum is shown in the following experiment.

The red blood cells were sedimented from a blood broth culture of *B. influenzae* by slow centrifugation; the supernatant culture fluid was again centrifuged and the sedimented bacteria were washed three times in large volumes of sterile salt solution to remove any trace of X substance adherent to them. The washed bacilli were resuspended in salt solution to the volume of the original culture and the relative growth capacity of the washed and unwashed bacilli from blood broth was tested in plain bouillon containing yeast extract in 10 per cent concentration.

The facts recorded in Table IV and substantiated in repeated experiments justify the assumption previously made that in addition to the V substance in blood which finds its analogue in yeast extract, there is also present another substance (X) equally essential to growth

TABLE IV.

*Relative Growth Capacity of Washed and Unwashed Influenza Bacilli from Blood Culture in Yeast Extract Broth.*

Inoculum.	10 per cent yeast extract broth (V) inoculated with.	
	Washed bacilli from supernatant fluid of blood broth culture (no X).	Unwashed bacilli from supernatant fluid of blood broth culture (X present).
cc.		
0.1	—	++
0.05	—	++
1 loop.	—	—

Controls: 0.1 cc. of supernatant blood broth in plain broth without yeast extract showed no growth. 1 loop of washed bacteria on blood agar yielded good growth.

of *Bacillus influenzae*. This accessory X substance, to the lack of which in media hemophilic bacteria are peculiarly sensitive, is capable in extraordinarily small quantities, such for instance as may be carried over in a single inoculum from blood broth, of supplying the necessary growth conditions. Although this second substance, the so called X factor, can function in minute amounts, it is unable by itself to induce growth, for, as shown by the controls in Table IV, the same inoculum which yields growth in broth containing yeast extract fails to grow in plain broth alone, although in both instances the amount of X carried over in the inoculum is the same.

These two substances may be further differentiated by their relative susceptibility to heat. It is known that the clear fluid extracted from the coagulum of whole blood by short exposure to the temperature of boiling water supports growth of *Bacillus influenzae*. This blood extract, therefore, presumably contains both the X and the V substances, and its addition to plain media in small amounts suffices for growth of hemophilic bacilli. Since, by boiling for several minutes, substances can be extracted from blood which still possess the growth-stimulating properties exhibited by unheated blood, it is evident that the factors concerned in promoting growth are not destroyed by

TABLE V.

*Effect of Heat on the Growth Accessory Substances of Blood.*

Dilution of blood extract in plain broth.	Blood extract.* 10 min. at 100°C.		Blood extract.* Autoclaved at 120°C. for 30 min.	
	Without yeast extract.	With yeast extract, 0.5 cc. (V).	Without yeast extract.	With yeast extract, 0.5 cc. (V).
1:10	++	++	—	++
1:100	++	++	—	++
1:1,000	—	+	—	+
1:10,000	—	—	—	—
1:100,000	—	—	—	—

\* Prepared by placing a tube containing rabbit blood in boiling water for 10 minutes and using the clear extracted fluid after removal of coagulated material by centrifugation.

short exposure to this temperature. In studying the effect of heat on the growth-stimulating action of tomato and yeast extracts (Table II) it was found that boiling for at least 10 minutes did not appreciably impair their action, while exposure in the autoclave to 120°C. for 30 minutes greatly diminished or completely destroyed their potency. If blood, therefore, contains a substance analogous in its behavior to the vitamine-like principle extracted from yeast, then blood which has been autoclaved should fail to support growth of *Bacillus influenzae*, since the V factor would be destroyed under these conditions. On the other hand, if the X factor of blood is stable to heat, then the autoclaved blood should be reactivated by the

addition of the fresh V substance in yeast extract. To test the validity of this assumption the experiment presented in Table V was carried out.

From Table V it is evident that the accessory substances in blood essential to growth of hemophilic bacilli are not destroyed by exposure to 100°C. for 10 minutes, since extracts prepared from boiled blood are active in promoting growth. It is further apparent that these same blood extracts after autoclaving for 30 minutes at 120°C. are no longer capable by themselves of supporting life of the bacilli. The experiment further demonstrates the interesting fact that the substance or substances in blood which are destroyed by excessive heat can be replaced by the addition of the growth-stimulating principle extractable from yeast. An analysis of these observations on the heat sensitiveness of the growth-promoting substances in blood indicates clearly that differences exist in thermostability on the basis of which it is possible to separate these two factors. The X factor, so called, is heat-stable and unaffected in action by exposure to steam under pressure at 120°C. for  $\frac{1}{2}$  hour. On the other hand, the so called V factor of blood, like the similarly reacting substance in extracts of yeast, is relatively more labile and is destroyed at the autoclave temperature.

Before passing to the question of the distribution of these growth accessory substances in the various fractions of blood, it is well to emphasize the peculiar sensitiveness of the hemophilic bacilli to a lack of either of these two factors in media, and the relative differences in the effective amounts of each. It has been pointed out (Table IV) that in a small inoculum from blood media, a sufficient amount of the X substance may be carried over to cause growth in blood-free broth, providing yeast extract, which supplies the V factor, is also added. In other words, mere traces of the X substance in the presence of this vitamine-like principle suffice for growth, but neither separately can function even when present in excess. This dual action, and the relative amounts of each factor necessary, particularly the minimal effective dose of X, are important in the technique of determining the presence or absence of either factor, since it is possible to carry over with the inoculum enough of the particular factor lacking in a test medium to permit growth. The nature of the seeding, therefore, must

be carefully chosen so that the culture from which it is taken shall contain only a minimum of the particular factor sought for in any given medium or extract. For example, when testing for the presence of the V factor in a given extract or medium, the seeding should be made from the supernatant fluid of a blood broth culture, since in this instance an effective amount of the X substance is carried over with little or no V factor. On the other hand, in demonstrating the presence of the X substance in blood or a derivative of blood, the medium in question should be inoculated from a yeast extract broth culture derived as above, since under these conditions a minimal and ineffective amount of X is transferred in the inoculum. In testing for the X substance an excess of the V factor should always be added in the form of yeast extract, or its equivalent.

*Distribution of the Growth Accessory Substances in Blood and Blood Derivatives.*—A comparative study of the distribution of these growth accessory factors in blood and blood derivatives was made by determining the presence or absence of growth of *Bacillus influenzae* in plain broth enriched with graduated amounts of ascitic fluid, serum, blood extract, and solutions of laked red blood cells and of crystalline hemoglobin. The sterile ascitic fluid, untinged with hemoglobin, was obtained from a patient suffering from cirrhosis of the liver and had been stored without antiseptic in the ice box for several months prior to use. The serum, blood extract, and solution of laked blood cells were prepared from freshly drawn defibrinated rabbit blood. The serum was separated from sterile defibrinated blood by repeated centrifugation, care being taken to obtain a specimen with no visible traces of blood pigment. The blood extract consisted of that fraction of whole blood expressed from the coagulum after boiling for 10 minutes, and separated from the coagulated proteins by prolonged centrifugation.

The solution of laked red blood cells was made as follows: The red cells from 20 cc. of sterile defibrinated blood were removed by centrifugation, washed three times in sterile salt solution (50 cc. each time), and taken up in sterile distilled water to the original volume of blood. After laking, the cell residue was sedimented by centrifuging and the clear supernatant fluid pipetted off and used as hemoglobin solution. By gasometric analysis 1 cc. of this solution contained 0.1 gm. of

hemoglobin. The crystalline hemoglobin<sup>3</sup> was prepared by the method of Welker and Williamson (11) from ox blood. The crystalline hemoglobin, as is usual with dry preparations, had lost its oxygen-carrying capacity. A water solution of the crystals (10 per cent by weight) was rendered sterile by passage through a Berkefeld filter (N).

TABLE VI.

*Distribution of the Growth Accessory Substances V and X in Blood and Blood Derivatives.*

Dilution in plain broth.*	Source of accessory substances.									
	Ascitic fluid.		Serum.		Blood extract.		Solution of laked red cells. †		Solution of crystalline hemoglobin. ‡	
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
1:2	—	+++								
1:5	—	+++								
1:10	—	—	—	+++	+++	+++	+++	+++	—	+++
1:100	—	—	—	+	+++	+++	+++	+++	—	+++
1:1,000			—	+	—	+	+	+++	—	+++
1:10,000			—	—	—	—	—	+++		+++
1:100,000			—	—				+\$		+++
1:200,000										+

\* All tubes inoculated with 0.05 cc. of an 18 hour yeast extract broth culture of *B. influenzae*.

† Contained 10 gm. of hemoglobin per 100 cc.

‡ Contained 10 per cent crystalline hemoglobin.

\$ Represents a final concentration of 1:1,000,000 hemoglobin.

|| Represents a final concentration of 1:2,000,000 crystalline hemoglobin by weight.

The ascitic fluid, serum, and blood derivatives prepared as described above were added to plain infusion broth in the dilutions indicated and inoculated with 0.05 cc. of an 18 hour yeast extract broth culture of *Bacillus influenzae*. Growth was controlled by subculture on blood agar and by second transfer to yeast extract broth alone.

<sup>3</sup> We are indebted to Dr. J. P. Peters, Jr., of the Hospital of The Rockefeller Institute for the preparation of crystalline hemoglobin used in these experiments.

An analysis of Table VI reveals certain facts concerning the distribution of the growth accessory factors in blood and blood derivatives. In the first place, it is evident that *Bacillus influenzae* will not grow in serum in concentration as high as 10 per cent in broth or in ascitic fluid diluted with equal volumes of broth. In the presence of yeast extract, however, growth occurred under otherwise identical conditions in ascitic fluid broth 1:5 and in serum broth 1:1,000. Secondly, blood extract and hemoglobin solution freshly prepared from laked red cells in broth were able to stimulate growth in dilutions as high as 1:100 and 1:1,000 respectively. The addition of yeast extract to broth containing these blood derivatives permitted growth in even higher dilutions; namely, 1:1,000 of blood extract, and 1:100,000 of the solution of laked cells. Finally, the addition of a solution of crystalline hemoglobin to broth in concentrations equivalent to the solution of laked red cells failed to yield growth, while in dilutions equivalent to 1:200,000 of laked cells the same solution of crystalline substance afforded excellent growth when yeast extract was added. It should be noted that for purposes of comparison the concentrations are expressed in Table VI in terms of dilution of the original body fluids or their equivalents. However, since in the solutions of laked cells and of crystalline hemoglobin the content of this substance by weight is 10 per cent, the actual dilution of hemoglobin sufficing for growth is, therefore, ten times greater than that recorded in the protocol. In terms of actual amount of crystalline hemoglobin by weight, these figures represent concentrations of 1:100 and 1:2,000,000 respectively.

In attempting to determine the distribution in blood of the so called growth accessory factors V and X, it must be borne in mind that the methods employed are not strictly comparable and that the results are merely relative and of necessity must vary with each individual specimen of serum or blood. Nevertheless, the growth differences are sufficiently great to make it apparent that the greatest concentration of both these substances is associated with the cell fraction of the blood, and that serum and ascitic fluid, on the other hand, contain little or no measurable quantity of the vitamine-like principle and only relatively small amounts of the X substance, as is evident from the fact that by themselves they are separately incapable of support-



ing growth. The quantity of X substance in pure serum is relatively slight as compared with its abundance in solutions of laked cells and crystals of hemoglobin. This fact makes it seem not unlikely that the red blood cell is the source of this substance and that the presence of X in serum or ascitic fluid is purely accidental, due to conditions which permit its escape from the blood cells. The activity of this substance in crystalline hemoglobin in dilutions as great as 1:2,000,000 by weight is strong evidence that it is some constituent of the red blood cell which may function as a catalytic agent. It must be borne in mind that the detection of the X substance by itself in solutions of crystalline hemoglobin, in ascitic fluid, and in serum is made possible only when these are used in combination with vitamine-containing extracts from yeast or from other extraneous sources, since the X substance alone is inactive and unable to support growth without the complementing V factor.

In attempting to interpret the facts brought out in Table VI, it is of further interest to observe the distribution and relative concentration of V factor, or vitamine-like principle, in the various fractions of blood and blood derivatives. Just as the X substance appears to be intimately associated with the cell fraction of blood, so the V factor is apparently found in greatest concentration in the blood corpuscles. Because of the greater susceptibility of the V factor to heat and chemical manipulation, and owing to the fact that it is present in blood in lower concentration than the X substance, this vitamine-like principle is active only when the intact red cell and solutions or extracts of these cells have not been subjected to untoward conditions. For example, the chemical procedures incident to the preparation of crystalline hemoglobin cause complete loss of this factor, while the more stable X substance remains unimpaired. Like the V factor, extractable from yeast and vegetable cells, the corresponding substance in blood extracts withstands boiling for at least 10 minutes, but is destroyed by exposure to 120°C. for 30 minutes, neither of which procedures, however, interferes with the peculiar properties of the X substance in blood. In the specimen of blood extract used in this experiment, the V factor exerted its growth-stimulating action in dilution of 1:100, and in the unheated solution of laked cells, in which the hemoglobin was physiologically active as shown by its oxygen-

carrying capacity, the V factor was present in dilutions as high as 1:10,000. On the other hand, as already noted, solutions of crystalline hemoglobin were devoid of vitamine-like property and required the addition of yeast extract to supplement the X substance present.

#### DISCUSSION.

As already pointed out, the importance of growth accessory substances in the cultivation of bacteria has been appreciated by numerous observers, and in the case of the hemophilic bacilli has been emphasized particularly by Davis. The foregoing experimental data are presented, therefore, not merely to direct attention to the relation of these substances to the growth of *Bacillus influenzae*, but rather with the hope that as these studies progress, they may furnish the basis for a more accurate understanding of bacterial nutrition, and that the principles involved may find wider application in the cultivation of organisms other than those of the hemophilic group.

In preceding papers (1, 12) it has been shown that the substances requisite for growth of *Bacillus influenzae* can be supplied from a source other than blood. The mucoid material elaborated during growth of certain bacteria, together with the dead bodies of these organisms in hemoglobin-free broth, has been found to furnish the accessory substances necessary for the cultivation of the hemophilic bacilli.

The present paper concerns itself with an attempt to analyze the accessory factors in blood which have to do with the peculiar nutritional requirements of the hemophilic bacteria. It is shown that the growth accessory substances in blood involve two distinct and separable factors, neither of which alone suffices to stimulate growth. One of these factors is analogous in its behavior to substances belonging to the class of so called vitamins, and because of this similarity is referred to in the text as the V factor. The other factor is less easily defined and is spoken of as the X substance. On the basis of relative differences in susceptibility to heat, these two factors may be separated one from the other. The vitamine-like principle in blood is destroyed by exposure to a temperature of 120°C. in the autoclave, while the X substance resists heating under these conditions. These substances, however, remain unimpaired in blood sub-

jected to the temperature of boiling water for 10 minutes. Extracts expressed from the coagulum of boiled blood contain both these factors and are capable, therefore, of supporting growth of *Bacillus influenzae*. On the other hand, autoclaved blood contains only the more stable X substance and is incapable by itself of stimulating growth unless reactivated by the addition of the V factor from another source. That the vitamine-like substance in blood can be supplied from other sources is shown by the fact that extracts of yeast, tomatoes, green peas, and beans possess the property of reactivating an otherwise inert medium containing only the X substance.

Furthermore, it is shown that the addition to plain broth of active extracts of yeast or vegetable cells in dilutions as high as 1:1,000 suffices to initiate growth of *Bacillus influenzae* when seeded from blood media. However, the fact that cultivation cannot be continued for more than one or two transfers in yeast extract broth alone suggests that in the first instance some of the X substance is carried over in the inoculum from the original blood culture in an amount sufficient to supplement the yeast broth and that growth fails in succeeding cultures because this X factor is either exhausted by growth or lost by dilution on subsequent transfer.

Moreover, study of the relative distribution of these two factors in the constituents of blood demonstrates the fact that they are present in greater concentration in the cellular elements than in the serum or plasma. The facts so far acquired indicate that the red blood cell is the carrier of the vitamine-like principle and that this substance, like its analogue in yeast cells, is intracellular in nature and can be extracted by the methods described. While the chemical nature of this substance is not known, it is presumably analogous to the so called vitamins. It has been found that similar growth-accelerating substances capable of replacing the V factor in blood can be extracted from bacterial and yeast cells and from fresh vegetables. These extracts contain but little nitrogen, they are destroyed by autoclaving, they are water-soluble, they pass a Berkefeld filter, but are absorbed readily from heated solutions by bone charcoal.

The experimental data recorded above indicate that in blood at least the X substance is intimately associated with or a derivative of hemoglobin. The fact that the X factor in crystalline hemoglobin

can function in dilutions as high as 1:2,000,000, when supplemented by an excess of the V factor from yeast extract, suggests that it may act as a catalytic agent. Further observations on the nature of the X substance in blood and its presence in material other than animal tissue will be presented in a subsequent paper.

#### CONCLUSIONS.

The hemophilic bacteria of which *Bacillus influenzae* serves as a type require for their growth two distinct and separable substances, both of which are present in blood and neither of which alone suffices. These substances are (a) a vitamine-like substance which can be extracted from red blood corpuscles, from yeast, and from vegetable cells, which is relatively heat-labile and absorbed from solution by certain agents; (b) a so called X substance which is present in red blood cells, is heat-stable, and acts in minute amounts.

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# STUDIES ON THE D'HÉRELLE PHENOMENON.\*

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PLATES 5 AND 6.

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## INTRODUCTION.

About 3 years ago d'Hérelle<sup>1</sup> found that stools of patients recovering from bacillary dysentery contain a filterable substance which is able to dissolve cultures of the Shiga bacillus and that a few drops of the dissolved culture reproduces the same phenomenon upon addition to another culture, and so on, indefinitely. Through these different passages the lytic property, instead of decreasing by dilution, is on the contrary increased and retains its activity even after several years.

Such a continuous transmission of the lytic property occurs only if the transfer is made into living cultures of the Shiga bacillus. Hence d'Hérelle concluded that the lytic agent is a filterable virus parasitic on the Shiga bacillus. To this supposed virus he has given the name of bacteriophage. He discovered similar bacteriophages for *B. coli*, *B. typhosus*, *B. paratyphosus* A and B, and some other bacilli.

Salimbeni<sup>2</sup> claimed to have isolated the bacteriophage, which he described as a myxameba possessing spores so minute that they are capable of passing porcelain filters.

Kabéshima,<sup>3</sup> on the other hand, denies the living nature of the bacteriophage and considers it merely as a catalyzer secreted by the leucocytes of the infected intestine and capable of activating a lytic proferment present in the body of the microbes.

The observations of Bordet and Ciuca<sup>4</sup> also make very doubtful the parasitic nature of the bacteriophage. These authors injected guinea pigs intraperitoneally

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\* Most of the findings here reported have already been presented as preliminary notes in *Compt. rend. Soc. biol.*, 1921, lxxxiv, 275, 750, 751, 753, 755.

<sup>1</sup> d'Hérelle, F., *Compt. rend. Acad.*, 1917, clxv, 373; 1918, clxvii, 970; 1919, clxviii, 631; *Compt. rend. Soc. biol.*, 1918, lxxxi, 1160; 1920, lxxxiii, 52, 97, 247.

<sup>2</sup> Salimbeni, *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1545.

<sup>3</sup> Kabéshima, T., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 219, 471.

<sup>4</sup> Bordet, J., and Ciuca, M., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 1293, 1296.

three times at 5 day intervals with cultures of *B. coli*. 1 day after the last injection the peritoneal leucocytic exudate exhibited the properties of the bacteriophage of d'Hérelle; namely, a continuous transmission of lytic action.

Bordet and Ciuca also observed that a culture of *B. coli*, once dissolved by an immunized exudate and then filtered, is able either to dissolve a second culture of *B. coli* or to inhibit its growth in broth. But neither this dissolution nor the inhibition is absolute, since a few organisms always resist the dissolution and multiply, although slowly. The latter bacilli are distinguished from the original culture by certain characteristics: they resist the lytic agent but have now themselves acquired the lytic property and become lysogenic, or capable of inducing dissolution in a culture of normal *B. coli*. Moreover, when planted on slanted agar a mucoid, sticky culture results; also they are less phagocytal and more virulent for guinea pigs than the normal culture from which they were derived. All these properties are preserved even after passage through animals.

Thus there seems to arise under the influence of the lytic substance a race of bacilli which is adapted to this substance and is characterized by new and transmissible properties such as the increased virulence. Bordet and Ciuca call this race "*modified B. coli*," and they infer that under the influence of the peritoneal exudate mentioned a variation of the colon bacilli occurs in the sense that they now secrete an autolysin which dissolves their own cells, with the exception of a few resistant organisms which survive and continue to produce the lytic secretion. Hence Bordet and Ciuca conceive the phenomenon to be that of a transmissible microbic autolytic property.

The facts as outlined are of fundamental importance, since they relate not only to the problem of the lysis itself but also to such disputed questions as the appearance of new races, the heredity of acquired characteristics, and the nature of virulence.

### *Influence of Hydrogen Ion Concentration.*

We first established the influence of the hydrogen ion concentration on the lytic action<sup>5</sup> which, as is known, manifests itself in two ways, either in inhibiting the growth of *Bacillus coli* in freshly planted broth cultures or in dissolving a culture already grown. As the former phenomenon is more easily observed than the latter, the inhibiting action of the lytic agent will be chosen as a criterion in the following experiments.

*Experiment 1.*—Two tubes of plain broth were seeded with the same small quantity of *B. coli*; i.e., with 1 drop of a 24 hour broth culture. Immediately

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<sup>5</sup> We are indebted to Dr. Bordet for a strain of *B. coli* with which he carried on his studies, together with a quantity of the corresponding lytic agent. The experiments were conducted with this material.

afterward 10 drops of the lytic agent were added to the second tube. In this condition, it is known that the first tube grows normally while the second remains perfectly clear for a certain period, which can be easily measured.

We have repeated this experiment with broths of varying hydrogen ion concentrations and observed that the inhibition is markedly influenced by the reaction of the medium; it is faint in a slightly acid (pH 6.8), neutral (pH 7), or even slightly alkaline broth (pH 7.4), but much stronger in a more alkaline broth (pH 8 or 8.5).

In a slightly acid broth (pH 6.8) containing 10 drops of lytic agent, *Bacillus coli* grows after 2 to 3 hours almost as well as in normal broth. But this early growth disappears very quickly by dissolution. After a few hours, however, a renewed growth begins and this time develops normally, reaching its maximum after 24 to 36 hours. Then another dissolution occurs, but this process is slow and incomplete. On the following day the culture becomes somewhat more opaque again.

Hence the impression arises of a succession of waves of growth and redissolution, at each wave of growth *Bacillus coli* becoming more resistant.

On the other hand, in a highly alkaline broth (pH 8.5) the inhibition is much more striking and it is only after 36 to 48 hours that growth appears.

Between these extremes we have observed all intermediate degrees, the inhibition increasing with the increase of the alkalinity.

### *Lysis and Microbic Variation.*

Bordet and Ciuca have clearly demonstrated that the lytic agent spread on the surface of a young culture of *Bacillus coli* on slanted agar clarifies the culture by dissolution, but that after a certain period of incubation one can detect a few irregular colonies which have the distinctive characteristics of the resistant *Bacillus coli* (Fig. 1, A). We observed a very similar picture by merely allowing the normal culture of *Bacillus coli* to age. An old agar slant of *Bacillus coli* shows a uniformly dull film on which appear very distinctly, here and there, small vitreous colonies. The first impression is that of a contamination, but these colonies are undoubtedly *Bacillus coli* and their distribution reproduces that of the resistant colonies

observed by Bordet and Ciuca (Fig. 1, *B*).<sup>6</sup> On transplanting the material of the desiccated film between the vitreous colonies, no growth occurs. The organisms originally in this material are now dead. On the other hand, if one of the vitreous colonies is planted in broth, a growth results which possesses a great resistance to the lytic agent. Therefore, by merely allowing the normal culture of *Bacillus coli* to age, we have realized an artificial selection of more resistant organisms.

At the same time we have had the good fortune to isolate from a subculture of the original strain a colony of organisms which are, on the other hand, extremely sensitive to the lytic agent. Hence two types of *Bacillus coli* were isolated—one very resistant (Strain R) and the other very sensitive (Strain S)—both artificially selected from the original culture in which they coexisted. The difference in the susceptibility of the two strains is illustrated in the following experiments.

*Experiment 2.*—1 drop of the lytic agent was spread on the 3 hour slanted agar growth of Type S. With the exception of only two or three colonies, an almost complete clarification occurred (Figs. 2, *A* and 3, *A* and *B*).

With Type R, however, the clarification was transient and soon afterward the path left by the drop was covered with a multitude of minute colonies (Fig. 2, *B*) which, by confluence, overgrew again the surface previously clarified (Fig. 3, *C* and *D*).

*Experiment 3.*—Broth of varying hydrogen ion concentrations and containing a few drops of lytic agent was seeded respectively with Types S and R. Type R grew luxuriantly, especially in acid medium (pH 6.8), while Type S showed no growth, even in an acid broth.

In addition to the differences in their susceptibility to the lytic agent, both types are distinguished by other characteristics. Type S, multiplying more rapidly than Type R, produces promptly in broth a supernatant film with a whitish band, consisting of microorganisms, adhering to the wall of the tube at the level of fluid surface; Type R shows the same details but only at a much later period of growth. Both types produce indole and ferment carbohydrates, with the exception of saccharose. The fermentation tests were made by means of stab cultures in semisolid agar and gave us the oppor-

<sup>6</sup> We have made similar observations with Shiga cultures.



tunity to observe another striking distinction. The growth of Type S remains close to the line of puncture (Fig. 4, *B*); on the contrary, Type R diffuses uniformly throughout the whole mass of agar (Fig. 4, *A*). This distinction is due to the difference in the motility of the two types of organisms; Type S is non-motile; Type R possesses, on the other hand, an active motility very much like that of the typhoid bacillus. This affords a very useful means of separating the two types when they are mixed: a stab culture is made in one arm of a U-tube containing semisolid plain agar; only the motile *Bacillus coli* diffuses to the other arm of the tube, from which, after a few hours, we are able to recover it in pure culture.

TABLE I.

Type S.				Type R.			
Guinea pig No.	Dose.	Dilution.	Results.	Guinea pig No.	Dose.	Dilution.	Results.
	cc.				cc.		
1	2	Undiluted.	Died after 26 hrs.	6	2	Undiluted.	Died after 10 hours
2	2	1:2	Survived.	7	2	1:2	" " 15 "
3	2	1:4	"	8	2	1:4	" " 12 "
4	2	1:10	"	9	2	1:10	Survived.
5	2	1:20	"	10	2	1:20	"

In general, *Bacillus coli* is not very virulent, and guinea pigs can withstand intraperitoneal injections of large doses of this micro-organism. Hence it is rather difficult to measure variation of virulence between different strains of *Bacillus coli*. However, in the following experiment, in which each type of this bacterium was employed, it will be noted that Type R is more virulent than Type S.

*Experiment 4.*—We injected into the peritoneal cavity of five guinea pigs respectively 2 cc. of a culture of Type S (grown in plain broth, pH 6.8, for 18 hours), using different dilutions, undiluted, 1:2, 1:4, 1:10, 1:20. Similarly five guinea pigs were injected with a culture of Type R. The results of this experiment are shown in Table I.

*Autopsies.*—Guinea Pig 1 (injected with *coli* S): The peritoneal cavity contained a purulent exudate with fibrinous clumps. Diffuse fibrinous membrane

covering the viscera, especially the liver. Microscopic examination of the exudate showed large numbers of leucocytes, a few containing phagocytosed bacilli. No free bacilli were visible (Fig. 5). The heart's blood yielded a sparse growth of Type S microorganisms.

Four other guinea pigs which died after injections of large doses of Type S have shown the same conditions, and in three of them cultures of the heart's blood remained sterile.

Guinea Pigs 6, 7, and 8 (injected with *coli* R): Only a faint fibrinous pellicle was noted on the surface of the liver. The peritoneal exudate was serosanguineous and on microscopic examination showed numerous bacteria and an occasional leucocyte (Fig. 6). The heart's blood yielded a profuse growth of Type R bacilli.

It appears from these observations that Type R is more virulent and less phagocytatable than Type S. The two types of *Bacillus coli* behave differently, therefore, not only on artificial media but also in the animal body, and, furthermore, retain their individuality even after one passage through a guinea pig.

In spite of the sensitiveness of Type S to the lytic agent, we have never observed a complete dissolution of all these organisms in a culture. Among the vast number of bacteria that are spread over an agar slant there are always a few—a dozen at least—that resist the lytic agent and form colonies. We have found that these resistant organisms were not a few individuals of Type R which had contaminated the culture of Type S; they were not motile. At least it proves that all the individuals which constitute a culture of Type S are not similar in so far as their susceptibility to the lytic agent is concerned. We assume that there are all degrees of resistance against the destructive agent, and if a few individuals can withstand the powerful action of the undiluted lytic agent there must be also a few so sensitive that they can be dissolved even by the lytic agent highly diluted, and that between these two extremes, intermediate degrees exist. This deduction is favored by the following experiment.

*Experiment 5.*—To a number of agar slants seeded with equal quantities of Type S bacilli and maintained for 3 hours at 37°C. was added 1 drop respectively of increasing dilutions of the lytic agent, as follows: undiluted,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , etc. As the concentration of the active agent diminished, the number of the resistant colonies increased. At a certain point the clarification of the surface was reduced to small irregular zones and finally to small, perfectly circular areas (Fig. 7).

These small areas of clarification represent the few individuals of *Bacillus coli* which are sensitive enough to be dissolved even in a very dilute solution of the lytic agent. Each of them, in turn, becomes a center of regeneration of the active substance, which, diffusing out at the same distance in all directions, produces a small, circular zone of clarification surrounded by a sort of halo.

*Relation to the So Called "Colonies of Bacteriophage."*

d'Hérelle, who first observed the localization of the dissolution when the lytic filtrate is very dilute, offers a different explanation for the formation of the small zones of clarification. He considers these areas as colonies of the invisible virus which he supposes to be responsible for the dissolution. When the filtrate is pure the individuals of the bacteriophage are so numerous that their colonies are confluent and produce a general lysis. But when the dilution is sufficiently high, each parasite is isolated and produces localized lysis.

He claims this peculiar aspect to be unquestionable proof of the living nature of the bacteriophage because he questions whether a diffusible substance can localize its action at certain points. As we have seen, we can explain this easily if we do not regard a culture of *Bacillus coli* as a homogeneous whole but as made up of organisms of varying resistance to the lytic agent. While d'Hérelle ascribed the localized dissolution to the dilution of the bacteriophage, we are inclined to look upon the dilution as accessory, and to search for the immediate source of the phenomenon in the relative resistance of the colon bacilli. If this is so, we should expect the similar production of small areas of clarification even with undiluted lytic agent on submitting to its action cultures of greater resistance. As will be shown later, this is precisely what we have often observed.

To sum up, the so called "colonies of bacteriophage" can be explained as well by the hypothesis of a lytic agent as by that of a parasite.

In the preceding experiment decreasing quantities of lytic agent were tested on a constant quantity of *Bacillus coli*. On the other hand, in the following experiment, increasing quantities of *Bacillus*

*coli* were submitted to a constant quantity of highly diluted lytic agent.

*Experiment 6.*—200 cc. of a 12 hour broth culture of Type S were centrifuged and to the sediment 2 to 3 cc. of broth were added. The mixture was then filtered through sterile cotton; in this way we obtained a perfectly homogeneous suspension with which the following dilutions were made:  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ . In large tubes was mixed respectively 1 cc. of each of these *B. coli* suspensions with 0.5 cc. of very dilute ( $10^{-5}$ ) lytic agent. Immediately afterward 1 cc. of each of these mixtures with 10 cc. of plain agar was plated in Petri dishes and incubated at  $37^{\circ}\text{C}$ . After 12 hours incubation, the surface of the plates was covered with a uniform pellicle of *B. coli* in which appeared small areas of clarification which were evenly distributed and easy to count.

If the lytic agent is a living organism and these areas are colonies of bacteriophage, the number of areas should be approximately the

TABLE II.

Dilution of lytic principle.	Dilution of <i>B. coli</i> .	Experiment 6. No. of spots.			
		a.	b.	c.	d.
$10^{-5}$	$10^{-4}$	69	—	—	25
$10^{-5}$	$10^{-3}$	249	56	70	220
$10^{-5}$	$10^{-2}$	469	167	187	800
$10^{-5}$	$10^{-1}$	328	211	332	508
$10^{-5}$	Undiluted.	142	95	255	320

same on all plates, since we have added to each the same amount of diluted filtrate. Moreover, since the size of a colony usually varies directly with the abundance of its food, and since *Bacillus coli* comprises the nutrient elements in this case, the size of the areas should increase with the quantity of *Bacillus coli*.

But the results of the preceding experiment show otherwise. The number of areas first increases, then reaches a maximum, and finally decreases, as the quantity of *Bacillus coli* is increased (Table II). The size of the areas instead of increasing, on the contrary, decreases.

These results favor the idea that the lytic agent is a diffusible substance. If the areas represent individuals of *Bacillus coli* so sensitive as to be dissolved even by a very weak lytic agent, the num-

ber of these sensitive individuals should increase with the concentration of the *Bacillus coli* emulsion, and the number of spots should then increase proportionately. This is what is noted at the beginning of the curve. But unfortunately a second phenomenon of opposite tendency interferes with the preceding one: when the lytic agent is added to the *Bacillus coli* suspension, it combines partially with proteins which have nothing to do with the dissolution and so decreases the dissolving action in direct proportion to the increase in quantity of *Bacillus coli*.<sup>7</sup> If, on one hand, the clarified areas increase with the increase of the *Bacillus coli* emulsion because the number of sensitive organisms is increasing, on the other hand, these areas tend to decrease because the activity of the lytic agent is decreasing. The combination of these two phenomena of opposite action should give us the curve that we have observed. The evidence at hand leads us to the hypothesis that bacteriophage is a diffusible, rather than a living substance.

#### *Non-Specificity of the Lysis.*

The lytic filtrate used in the preceding experiments was specific. While it is active on the *Bacillus coli* used in injecting the guinea pigs, it is without any action not only on other closely related species but also on other strains of *Bacillus coli*. But in the following experiments we have been able to extend the lytic action to other species.

*Experiment 7.*—With the aim of increasing our stock of lytic agent we added 1 cc. of the original filtrate to each of two flasks, one containing 25 cc. of a young broth culture (pH 8) of Type S and the other of Type R. Dissolution of the growth occurred. After 48 hours incubation, we filtered both cultures and thus obtained besides the original filtrate (Filtrate O), two new filtrates, Filtrate 1 (Type S) and Filtrate 2 (Type R).

With a sample of Filtrate 2 a very marked dissolution was observed<sup>8</sup> of Shiga bacilli, Flexner bacilli, Hiss Y type of Flexner bacilli, and also a strain of *B. coli*

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<sup>7</sup> This phenomenon is similar to the one observed by Loeb (Loeb, J., Artificial parthenogenesis and fertilization, Chicago, 1913, 145) on the parthenogenetic fertilization of eggs with small quantities of acids. Very dilute solutions of acid are able to fertilize a small number of eggs, but not a larger number. The acid in combining with the jelly of the eggs loses a certain part of its activity; it still retains enough efficiency when the eggs are not numerous, because the total quantity of jelly is small, but loses it completely, on account of the abundance of jelly, when the number of eggs is great.

<sup>8</sup> These observations were made by Dr. Martha Wollstein.

*communis* which was unattacked by the original filtrate. This observation led us to control the action of all three filtrates on agar slants, seeded with the following bacteria: Types S and R, both isolated from the original culture of Bordet and Ciuca, *B. coli communis*, *B. coli communior*, *B. dysenteriae* Shiga, *B. dysenteriae* Flexner, Hiss Y type of Flexner bacilli, *B. typhosus*, and *B. paratyphosus* A and B.

Filtrate O, the original lytic agent, had only a weak lytic action, limited to Types S and R; Filtrate 1 was similarly weak, but produced a few small areas of clarification on the three dysenteric bacilli; Filtrate 2, on the other hand, was extremely active and produced a complete dissolution of Type S, an almost complete dissolution of Type R and of the three dysenteric bacilli, and a complete clarification of the strain of *B. coli communis*. Only the *B. coli communior*, the typhoid, and both paratyphoid strains were unaffected.

Our supposition was to attribute this increase of the power of the lytic agent to the fact that Filtrate 2 was prepared with a more re-

TABLE III.

Filtrate.	<i>B. coli.</i>				Dysenteric bacilli.			Typhoid.	Paratyphoid bacilli.	
	Type S.	Type R.	<i>Communis</i> .	<i>Communior</i> .	Shiga.	Flexner.	Hiss Y.		A.	B.
O	+++	++	—	—	—	—	—	—	—	—
1	+++	++	—	—	+	+	+	—	—	—
2	++++	++++	+++	—	+++	+++	+++	—	—	—
3	++++	++++	+++	—	++++	++++	++++	+	—	—
4	+++	++	+	—	++	+++	+++	+++	—	++

++++ indicates complete clarification, no resistant colonies; +++, almost complete clarification, less than twelve resistant colonies; ++, moderate degree of clarification, many resistant colonies; +, only a few small areas of clarification; —, negative result.

sistant strain, Type R. The following experiment demonstrates that this is the actual condition and the use of a resistant strain gives a method of increasing the efficacy of the lytic agent and extending its action to the other species as yet refractory.

*Experiment 8.*—By allowing 1 cc. of Filtrate 2 to act for 48 hours on 25 cc. of a young culture of Type R bacilli, we have obtained a still stronger filtrate (Filtrate 3) which, fortunately, produced four or five minute areas on a slant of typhoid bacillus. Following this last indication, we mixed 25 cc. of a young culture of typhoid bacillus with 3 cc. of Filtrate 3, and after 48 hours incubation, obtained a filtrate (Filtrate 4) extremely lytic for typhoid, as well as for para-

typhoid B bacilli. At the same time, the action of Filtrate 4 on *B. coli* was diminished to a certain extent. Table III shows the activity of the different filtrates.

It is obvious that we can now multiply similar combinations by allowing one or another of our filtrates to act on a well selected strain as intermediary. It will be of interest to study the relation between different microbic species in this respect, and to note to what extent the lytic activity can be transmitted from one species to another.

#### CONCLUSIONS.

The inhibition produced by the lytic agent on the growth of *Bacillus coli* is greatly influenced by the reaction of the medium; it is faint in a slightly acid (pH 6.8) or neutral (pH 7) or even slightly alkaline broth (pH 7.4), but is much stronger in a more alkaline medium (pH 8 or 8.5).

We have isolated from the original strain of *Bacillus coli* two types of organisms; one (Type S) is sensitive to the lytic agent, the other (Type R) is much more resistant. These types are distinguished also by other characteristics: Type S grows quickly in artificial medium and is non-motile; Type R grows more slowly, is extremely motile, much less phagocytatable, and more virulent. Both types produce indole and ferment carbohydrates, with the exception of saccharose. Both types keep their individuality even after passage through a guinea pig.

We have also demonstrated that even a culture of a single type, Type S for instance, is not a homogeneous whole but is made up of organisms of varying resistance to the lytic agent; only a few are resistant enough to overcome the strong action of the undiluted lytic agent. On the other hand, only a few as well are sufficiently sensitive to be dissolved even by very dilute lytic agent.

This explains why dilute lytic agent spread on an agar plate seeded with *Bacillus coli* confines its action only to certain places and produces the small round areas of dissolution that d'Hérelle considered as "colonies of bacteriophage." Moreover, we have observed the same localized action even with non-dilute lytic agent when submitting to its action cultures of greater resistance.

Our original lytic agent was found to be specific; it acted exclusively on the *coli* with which the guinea pigs were injected. By allowing this original lytic principle to act on broth cultures of our two types of *Bacillus coli*, we have obtained two new filtrates. The first, resulting from dissolution of the sensitive Strain S, is specific as is the original filtrate. But with the second, obtained from the resistant Strain R, Dr. Wollstein has found a marked action on Shiga, on Flexner, and on Hiss dysentery bacilli. In consequence of this observation, we have been able, by a method of successive passages through appropriate strains, to extend the lytic power to other species, as typhoid and paratyphoid bacilli, and have obtained by this somewhat different technique results similar to those recently published by Bordet and Ciuca.

#### EXPLANATION OF PLATES.

##### PLATE 5.

FIG. 1. Tube A: Experiment of Bordet and Ciuca. A young culture of *B. coli* on slanted agar covered with lytic agent. Only a few organisms resist dissolution and produce irregular colonies. Tube B: Agar slant culture of *B. coli*, 6 weeks old. Note on the uniformly dull film of the desiccated culture a few hyaline colonies which have resisted desiccation (*coli* R).

FIG. 2. Experiment 2. 1 drop of lytic agent placed on 3 hour cultures of *B. coli* (Types S and R). Results after 6 hours incubation at 37°C. Tube A: Type S (sensitive). The path of the drop is free from growth. Tube B: Type R (resistant). The path of the drop is already covered with a great number of minute resistant colonies.

FIG. 3. The same experiment. Results after 24 hours incubation. Tubes A and B: *Coli* S. Only a few resistant colonies may be seen. Tubes C and D: *Coli* R. Numerous resistant colonies have overgrown the area previously clarified.

##### PLATE 6.

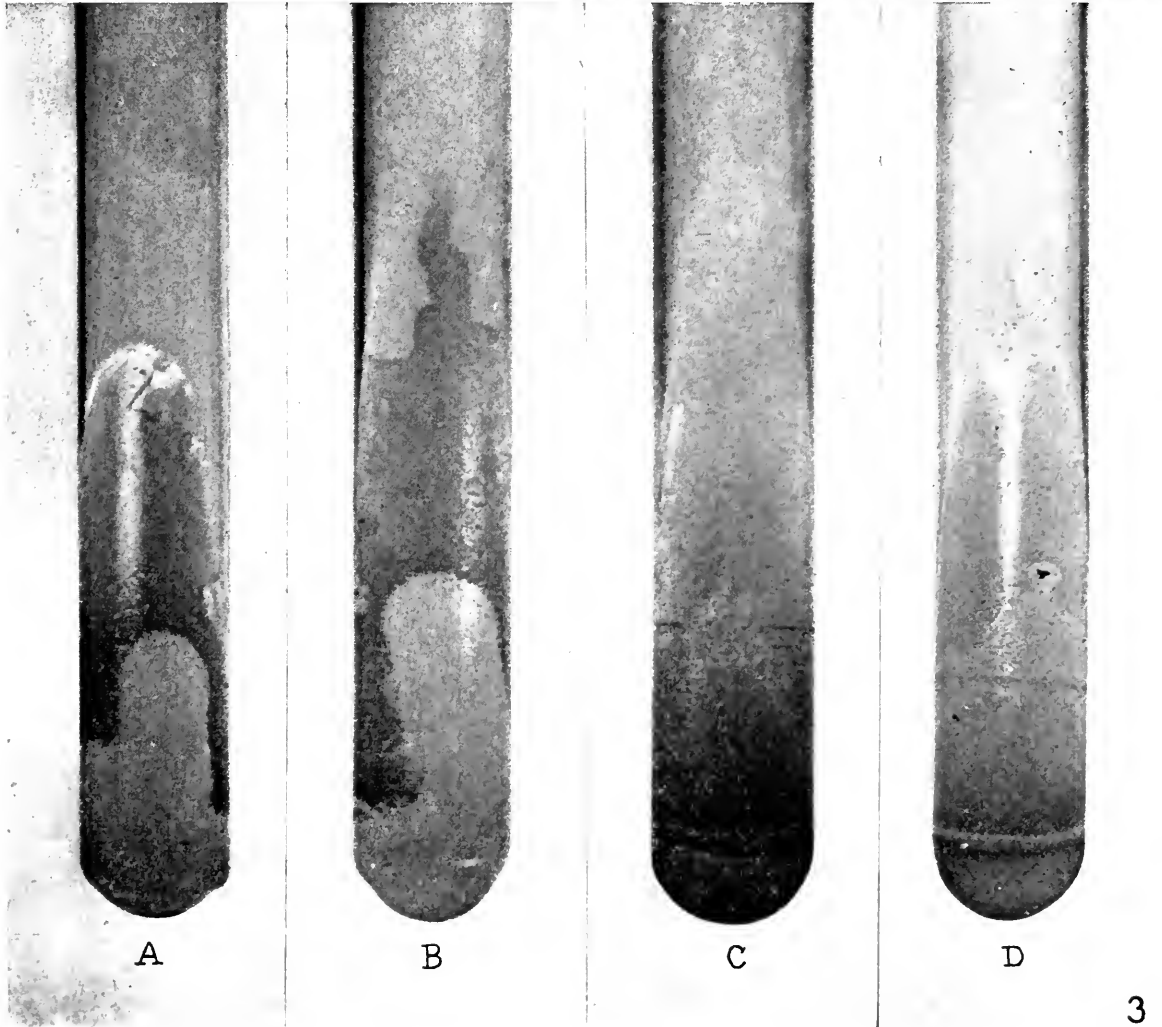
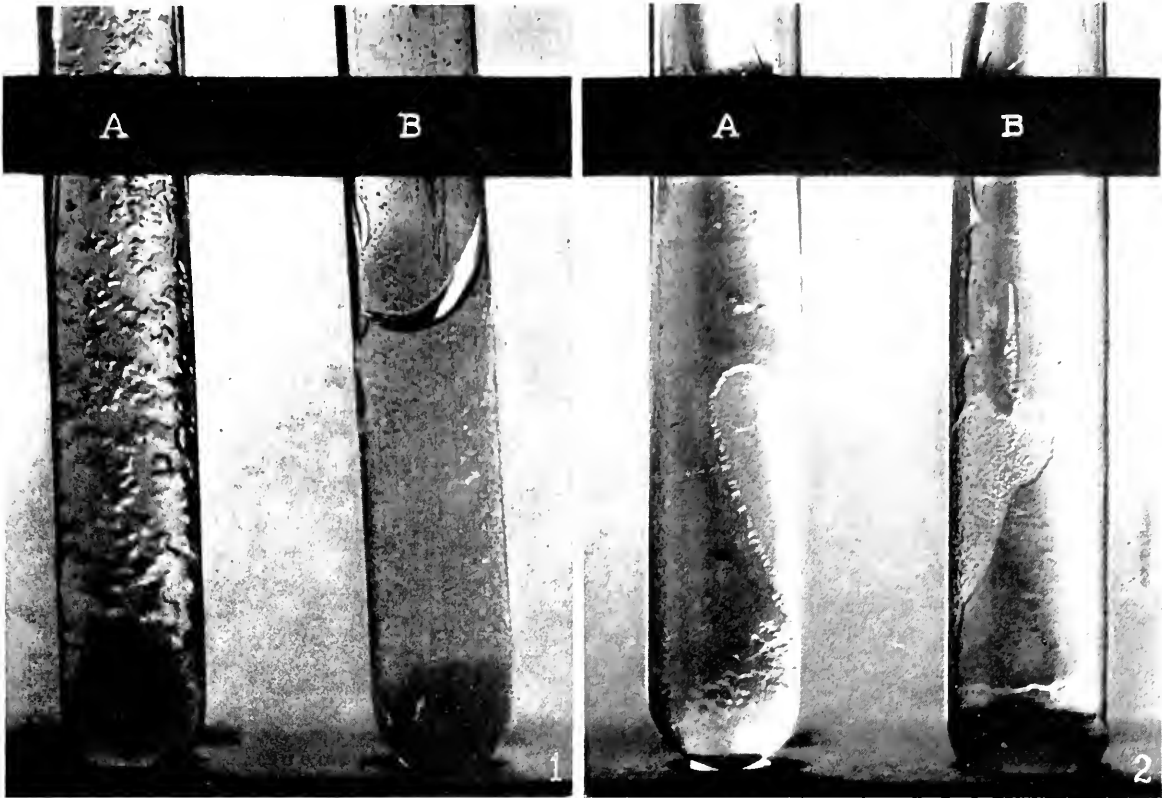
FIG. 4. Stab cultures of Types S and R in semisolid agar. Tube A: Type R (motile). The growth diffuses evenly throughout the whole mass of agar. Tube B: Type S (non-motile). The growth remains close to the line of puncture.

FIG. 5. Peritoneal exudate of a guinea pig dead after injection of *coli* S. Numerous leucocytes. No free bacteria.

FIG. 6. Peritoneal exudate of a guinea pig dead after injection of *coli* R. No leucocytes. Numerous free bacteria.

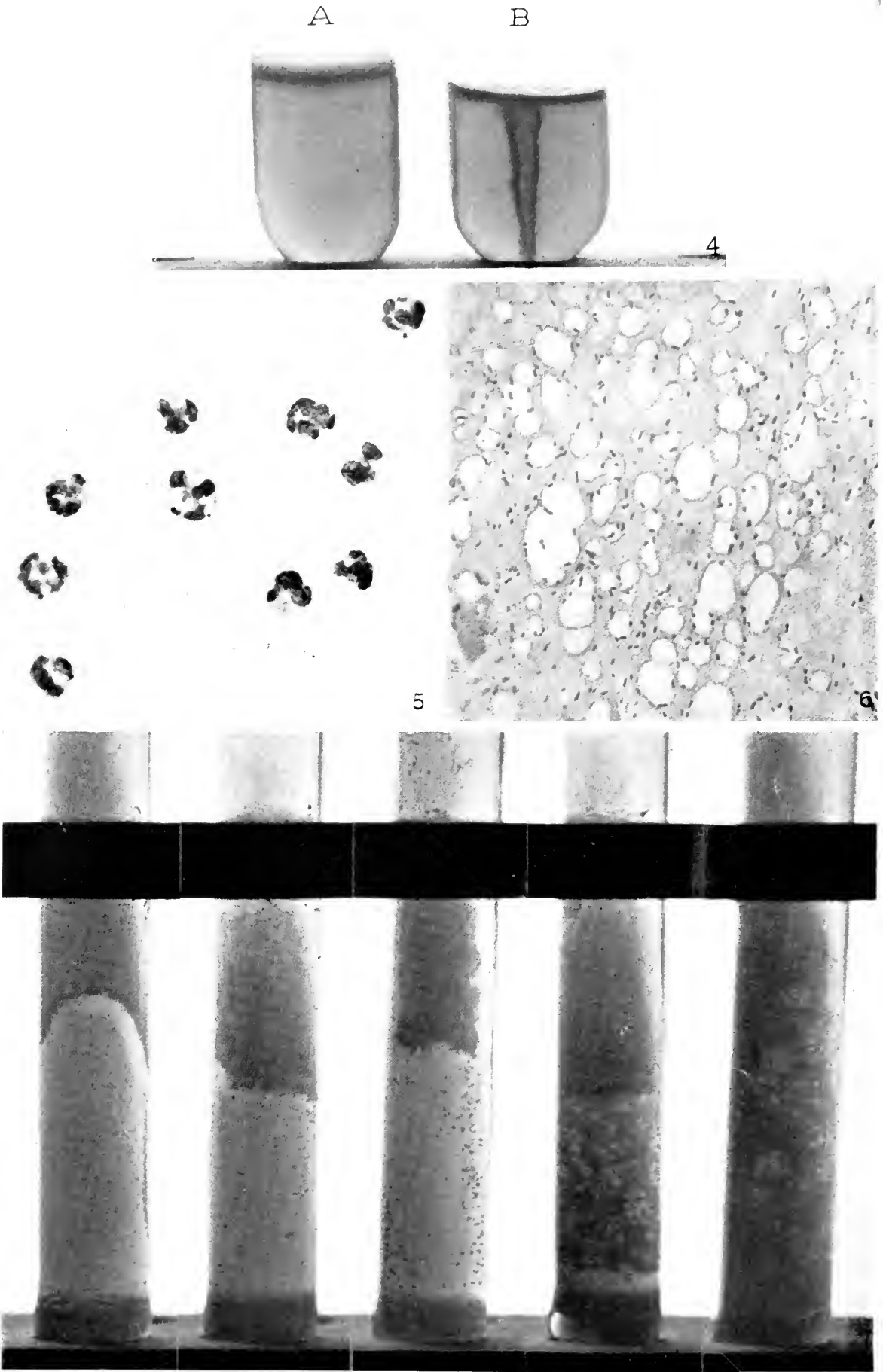
FIG. 7. Action of increasing dilutions of lytic agent on *B. coli* S.





(Gratia: d'Hérelle phenomenon.)





(Gratia: d'Hérelle phenomenon.)



## STUDY OF TRANSFUSED BLOOD.

### I. THE PERIODICITY IN ELIMINATIVE ACTIVITY SHOWN BY THE ORGANISM.

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It was hoped that some light might be thrown on the length of life of normal blood corpuscles and the mechanism of their removal from the circulation by the study of the elimination of transfused blood, which it is possible to make when the transfused blood is of a group unlike that of the recipient. This study has brought to light two facts, first, that the length of time that transfused blood remains in the circulation varies greatly; and second, that the elimination is not a continuous process but takes place in more or less cyclic crises, so that the responsibility for the disappearance of transfused blood from the circulation seems to rest more heavily on this cyclic activity of the body than upon the condition of the corpuscle.

The probable life of the blood corpuscle was reduced to 10 days or less by calculation based upon bile pigment output, made by Zoja and others, after the time of Ward-Muller, Quinck, von Ott, and Hunter, who from transfusion experiments in dogs concluded that the life of the normal corpuscle was from 14 to 26 days. The experimental results of Todd and White (1909-10) upon cattle, obtained by using an isohemolytic serum to separate out transfused blood, are in agreement with the shorter period. The work of Whipple and Hooper, however, who show that bile pigment is only in part derived from blood pigment, has made the shorter calculations based on bile pigment excretion untenable. The observations of Rous and Turner, who kept rabbit corpuscles *in vitro* for 14 days and then substituted them for a rabbit's own blood without any abnormal results, would also indicate a longer life of the corpuscles, as do my own results (1919, a) of the study of transfused blood in man.

As for the mechanism of blood elimination, we must undoubtedly give credit to some extent to phagocytosis, which plays a more or less important part in all

animals and appears, moreover, to be capable of extension under stimulation. Keyes finds in a wide range of animals that the physiologic destruction of the animals' own red blood corpuscles is accomplished by specialized fixed tissue phagocytes, which are confined largely to the liver or to the spleen. In birds, amphibia, and the lower mammals the phagocytic activity is most marked in the liver; in the higher animals it is most marked in the spleen. Rous and Robertson (1917) made quantitative estimates of the presence of phagocytes in the spleen of guinea pigs, cats, dogs, rabbits, *rhesus* monkeys, and man, and found that although in some of these animals phagocytic cells were present in numbers that might account for physiologic blood destruction, in the monkey and man these were few. Muir, in the study of phagocytosis in a rather large series of cases of empyema, smallpox, and pneumonia, found that phagocytosis was negligible in the bone marrow, but that in the spleen phagocytic activity was displayed against the native red blood corpuscles by cells of the splenic pulp and by certain hyaline leucocytes within the pulp. Carey, using intravenous injections of foreign red cells, and Downey, using intravenous injections of dye granules, found that under increased stimulation there is an increase in the number of cells which act as phagocytes. Downey believes that many "connective tissue" cells, may, under stimulation, become phagocytic. Carey finds under repeated stimulation an extension of phagocytic activity from cells of the spleen to those of the liver.

Rous and Robertson (1917) have recently given us knowledge of a second method of blood destruction, that of fragmentation in the blood stream with, it would seem, a filtering out of these fragments by the spleen. This they consider in man to have a greater importance than phagocytosis.

There are certain anomalous findings that suggest the possibility that a specific antibody mechanism is responsible for physiologic blood destruction. Todd and White's (1909-10) isohemolytic serum would not hemolyze ox corpuscles *in vitro* in the presence of ox serum unless guinea pig complement was added, but when injected intravenously into oxen produced massive hemolysis. Todd and White considered that this indicated the presence of some organ in the body which was able to produce the activating effect of guinea pig complement. Rous and Robertson (1918) found in some of their rabbits that had been receiving massive doses of rabbit blood that an isoagglutinin developed which caused agglutination of the corpuscles of the animals at room temperature. This agglutinin tended to appear in those rabbits which in the face of heavy blood injections developed an anemia. Davis and Macgregor both report evidence of erythrophagocytic bodies in meningitic patients, and in paroxysmal hemoglobinuria we have the isohemolysin which only unites with complement at low temperatures. Although one is tempted to speculate upon the possibility that physiologic blood corpuscle destruction is produced by some organ or organs of the body that form an antibody having lytic and opsonic qualities and so promoting fragmentation and phagocytosis, we have no evidence that any such antibody exists.

If we can draw a parallel between the elimination of transfused blood and normal blood destruction, the following study of transfused blood makes, I feel, a contribution to our understanding of blood destruction in that it would seem to connect the process with the activity of the endocrine gland system.

### *Method.*

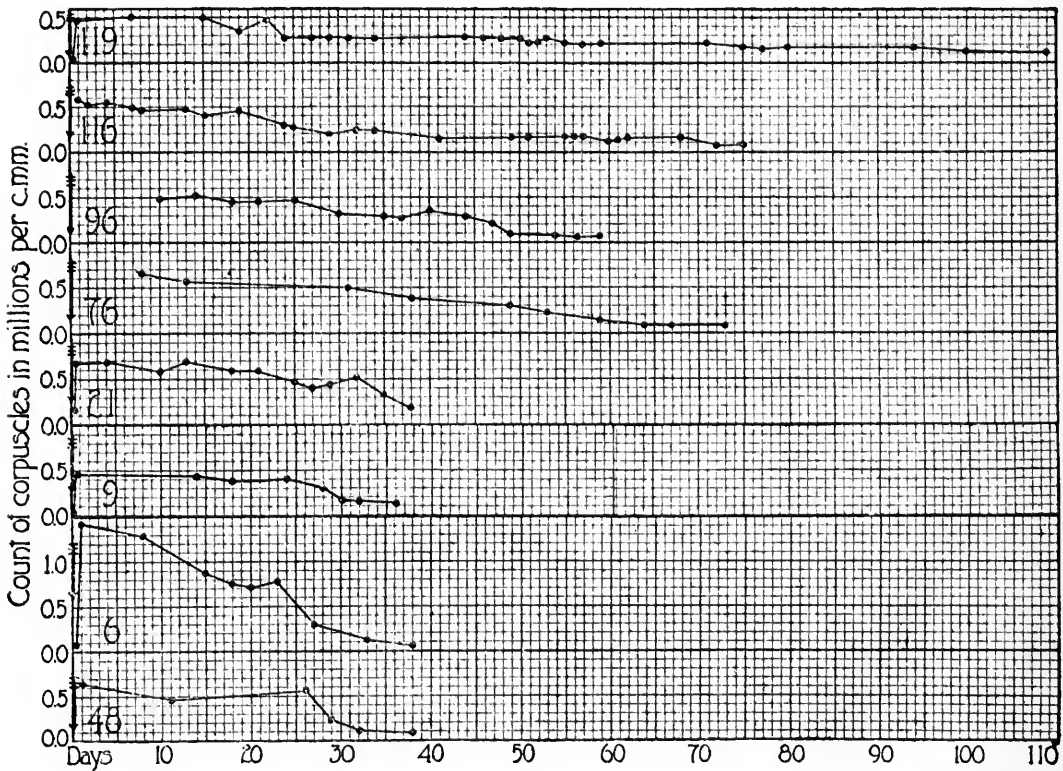
In the following work the elimination of transfused blood has been studied in individuals in Groups I, II, and III receiving Group IV transfusions. The method used was that published by me originally in *The Journal of Experimental Medicine* (1919, a), with certain modifications described in the *Medical Clinics of North America* (1919, b). It consists, in brief, of diluting the recipient's blood after transfusion with Group IV serum. This agglutinates the native corpuscles, leaving the transfused corpuscles free and capable of being counted in a hemocytometer. The dilution is made in a white blood-counting pipette, Group IV citrated serum being used as the diluent, and the mixture is expelled into Wassermann tubes. The tubes are incubated for 40 minutes with shaking, and allowed to stand at room temperature for from 15 to 30 minutes. A count is made by daylight, or, if it is necessary to use artificial light, care is taken to protect the corpuscles from its effect. All counts are made in duplicate.

Since by the use of this technique it is necessary that the transfused blood be of a different group from the recipient's blood it seemed more desirable, if it were feasible, to use the method of Todd and White (1909-10), which in cattle gives a differentiation between the blood of individuals. Patients who had received a series of from four to five 500 cc. transfusions were tested for hemolysin. Blood plasma from these patients was used both fresh without guinea pig complement and with guinea pig complement against corpuscles of the group to which the plasma belonged. Neither agglutinins nor hemolysins could be detected. This method is not applicable.

In harmony with the current conception of the behavior of native corpuscles, I assumed that transfused blood corpuscles would slowly deteriorate in the circulation and upon reaching a certain degree of degeneration either fragment or be phagocytosed, while their place was taken by new corpuscles. If this should be the case, on the injection of a normal blood into individuals not having blood disease one would expect a certain approximate equality in the length of time that expires before the transfused blood leaves the circulation, and by studying several curves of elimination to the end it seemed probable that a figure could be obtained that would roughly represent the length of life of the newest corpuscles injected.

## RESULTS.

In Text-fig. 1 are given the curves of elimination of transfused blood in the eight cases of individuals without blood disease which have been studied to complete elimination or to nearly complete elimination. The time taken for elimination was approximately 100, 72, 49, 63, 38, 30, 34, and 33 days.



TEXT-FIG. 1. Curves showing the variability of the time taken by individuals without blood disease to eliminate Group IV transfused blood. ↓ indicates a Group IV transfusion.

In these data the variation in the time taken to eliminate Group IV transfused blood is so great that no time can be given as the average length of life of the transfused corpuscles. Although slight individual differences in bloods can be noted with respect to resistance to hypertonic salt solution and other hemolysins, it would hardly seem probable that so great a difference in the time of elimination as that between 100 days and 30 days could be attributed to differences in resistance of the transfused bloods, to which dif-



ferences in resistance to hypertonic salt solution might be an index. It will be noted that the longest curve, 100 days (Case 119), was obtained in the case of a man in health, while the elimination time was only 28 to 30 days in the case of a very cachectic cancer patient (Case 48). No generalization, however, can be made from this, as a patient (Case 9) whose blood had returned to normal after hysterectomy eliminated the transfused blood in 28 to 30 days, and a patient who died with long standing infection (Case 95) had shown no definite sign of elimination of the transfused blood after 46 days. The protocols of these individuals are given below.

*Case 6.*—A woman, aged 35 years; weight 138 pounds. An operation for fibroid of the uterus was performed, followed by two transfusions because of severe hemorrhage.

*Case 9.*—A woman, aged 33 years, whose normal weight was 100 pounds, was transfused previous to subtotal hysterectomy. The convalescence was satisfactory.

*Case 21.*—A woman, aged 45 years, weighing 87 pounds, had common duct obstruction, and tertiary syphilis of the liver.

*Case 48.*—A woman, aged 48 years, weighing 127 pounds, had cancer of the breast with extensive glandular involvement. The menstrual periods had been regular up to February 7, 1919. There was no further menstruation until March 28, when the menstrual flow began and continued until April 23. If the menstrual periods had continued to be regular after February, they should have occurred March 7, April 4, and May 2. On April 5 a radical operation was performed, followed by a transfusion.

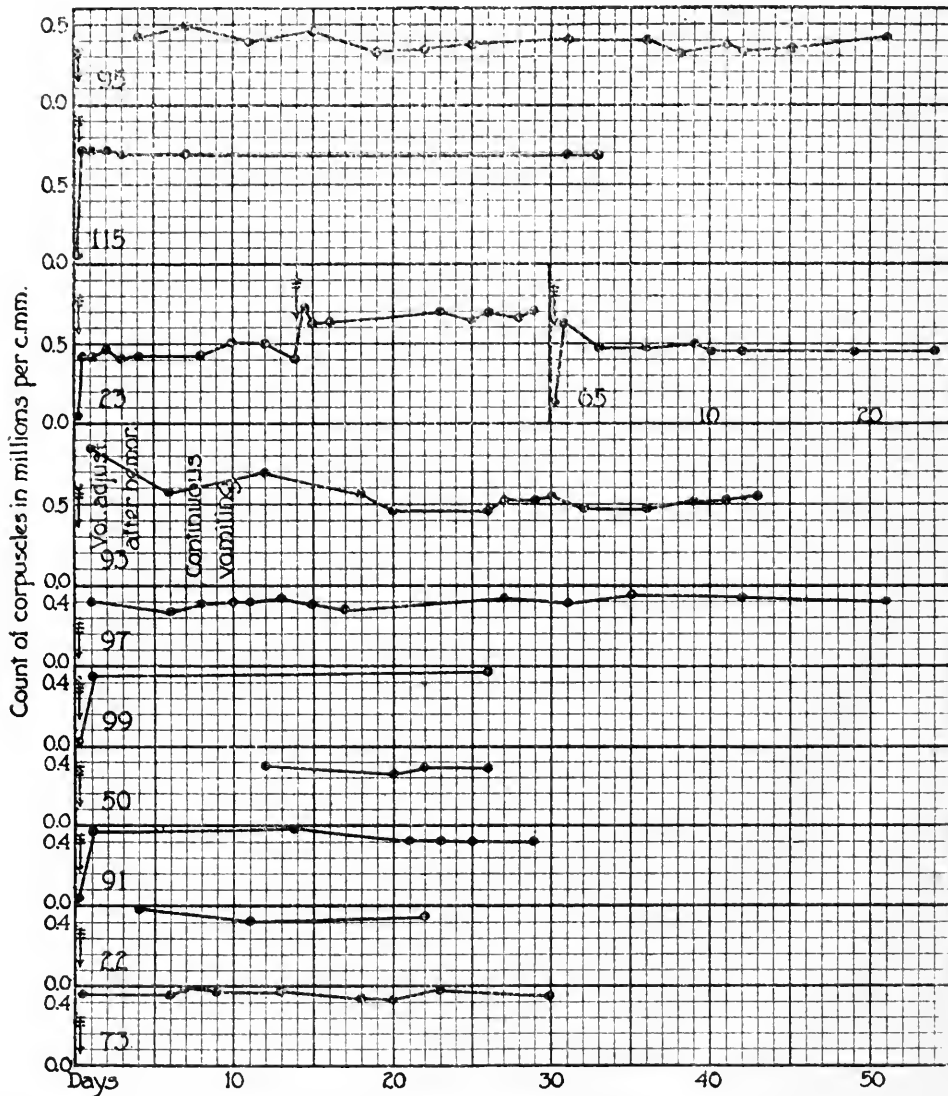
*Case 76.*—A woman, aged 24 years, had chondrosarcoma of the right ribs. A two-stage operation was performed for removal of the tumor. Each operation was followed by a Group IV transfusion of blood.

*Case 96.*—A woman, aged 40 years, had a malignant pelvic tumor. A total hysterectomy was performed, followed by a blood transfusion.

Cases 116 and 119 are given below.

With reference to the cyclic elimination of the transfused blood, my material brings out five points. (1) The curves of elimination of Group IV transfused blood do not show a gradual downward curve day by day, which would be expected if it is assumed that blood corpuscles gradually wear out and are replaced. When due allowance has been made for blood volume changes, it will be seen that the count stays on a level for a long time. (2) When the counts have been taken sufficiently close together the curve of elimination

makes a sudden drop to a lower level, at which it again remains for many days. There are longer periods of no elimination and shorter periods of elimination. (3) The nature of these drops is such that they are more likely to be due to an active destruction on the part of the body than to, what would be the other possibility, a coinci-



TEXT-FIG. 2. Curves showing that over a long period of time there is no elimination of transfused blood. ↓ indicates a Group IV transfusion.

dent spontaneous disintegration of the corpuscles. (4) Accompanying these drops in the count of the transfused blood, there is usually an activity of the bone marrow as indicated by a rise in the total blood count. (5) In women these drops are related to the menstrual cycle.

Text-fig. 2 illustrates the fact that over a long period of time there is no elimination of transfused blood. The protocols of these cases follow.

*Case 22.*—A man, aged 32 years, weighing 103 pounds, had duodenal ulcer and chronic appendicitis. A posterior gastroenterostomy and appendectomy were performed, followed by a transfusion.

*Case 23.*—A man, aged 57 years, weighing 139½ pounds, had pernicious anemia and was given a series of transfusions.

*Case 50.*—A man, aged 50 years, a farm laborer of powerful build, received a transfusion after appendectomy and gall bladder operation.

*Case 65.*—A boy, aged 11 years, weighed 63 pounds. A diagnosis of lymphatic leucemia was made. He was transfused.

*Case 73.*—A woman, aged 47 years, weighing 120 pounds, had pernicious anemia. She received a series of transfusions.

*Case 91.*—A woman, aged 53 years, weighing 137 pounds, with pernicious anemia, received a series of transfusions.

*Case 93.*—A woman, aged 43 years, was given a transfusion because of post-operative hemorrhage after gall bladder operation. On the 8th and 9th days after transfusion subcutaneous injections of saline solution were given because of continuous vomiting.

*Case 95.*—A man, aged 31 years, had an arteriovenous aneurysm between the popliteal artery and vein, resulting from injury by a bullet. After operation secondary hemorrhage developed, necessitating a transfusion 6 days later. On the 19th day after transfusion the leg was amputated for dry gangrene. On the 55th day following hemorrhage, a pelvic exploration was done. The patient died on the 57th day after transfusion.

*Case 97.*—A man, aged 42 years, weighing 155 pounds, had pernicious anemia and was given a series of transfusions.

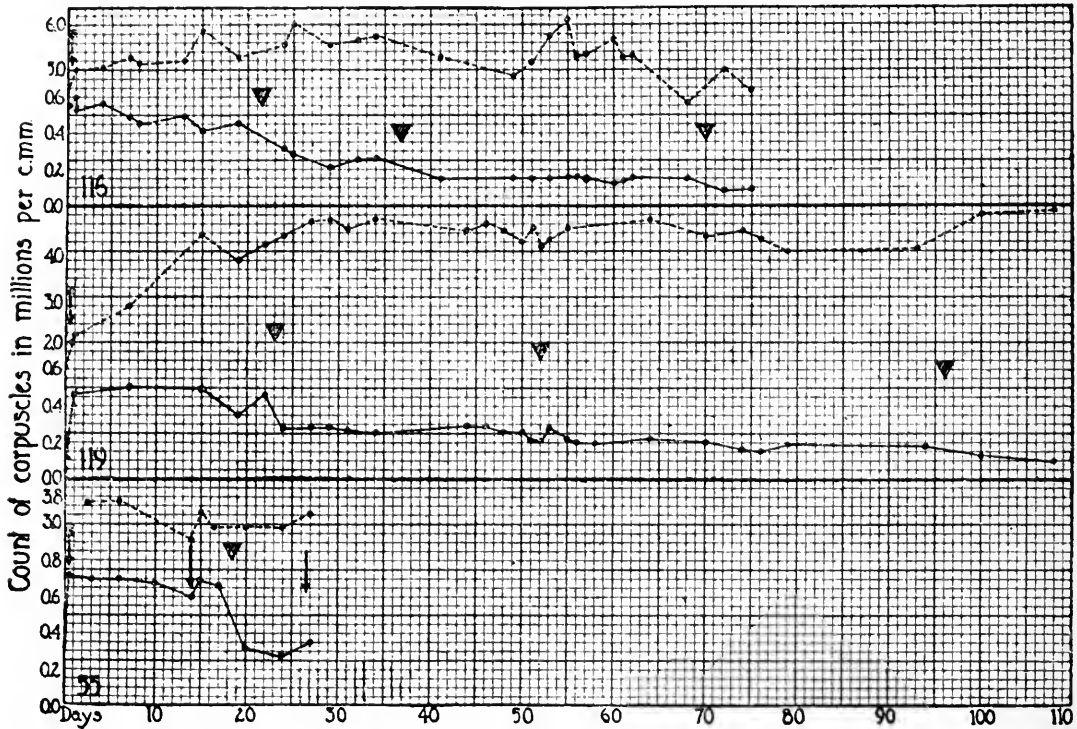
*Case 99.*—A girl, aged 17 years, weighing 100 pounds, was given a blood transfusion for secondary anemia due to malaria. Following medication there were no more chills.

*Case 115.*—A woman, aged 33 years, weighing 141 pounds, was given a blood transfusion January 26, 1920, because of secondary anemia. Normal menstruation occurred while the patient was under observation, January 26, February 27, and March 24.

The protocols of cases included in Text-fig. 3 are given below. In Cases 116 and 119 the tabulation of the blood counts, which are omitted in other instances to save the cost of reproduction, are partially given, as the length of time over which the study was made necessitated a reduction in the charts, which leaves them inade-

quate as evidence of the small changes which constitute the later drops in the count.

*Case 55.*—A woman, aged 51 years, whose menopause had occurred 2 years before, was completely jaundiced from common duct obstruction. There was bile in the urine.



TEXT-FIG. 3. Curves of elimination of Group IV transfused blood in two men, and a woman passed the menopause, showing that there are comparatively short periods of elimination and that these periods are irregularly cyclic. — Count of unagglutinated corpuscles. --- Total red blood count.  $\nabla$  indicates a Group IV transfusion;  $\uparrow$  a like group transfusion. The cross-hatched triangle calls attention to periods of elimination.

*Case 116.*—A man, aged 20 years, weighing 125 pounds, had been troubled with bleeding of the nose for the past 9 years. He had a bleeding time of 40 minutes and a coagulation time of  $5\frac{1}{2}$  minutes; platelets were 88,000, and hemoglobin 43 per cent. The diagnosis was hemorrhagic purpura. One transfusion was given January 29, 1920. On March 20 the hemoglobin was 80 per cent.

Length of time after transfusion.	No. of unagglutinated corpuscles per c.mm.
<i>days</i>	
0 (Transfusion with 500 cc. of Group IV citrated blood.)	
0	556,000
0	566,000
1	522,000
4	555,000
7	495,000
8	466,000
13	487,000
15	416,000
19	466,000
24	313,000
25	295,000
29	221,000
32	250,000
34	255,000
41	162,000
49	168,000
51	163,000
53	166,000
55	170,000
56	159,000
57	162,000
60	128,000
61	145,000
62	154,000
68	163,000
72	81,000
75	93,000

*Case 119.*—A man, aged 33 years, with a cystic tumor at the base of the tongue, came for examination because of sudden hemorrhage. The patient's blood condition rapidly returned to normal after transfusion and he was able to go to work.

Length of time after transfusion.	No. of unagglutinated corpuscles per c.mm.
<i>days</i>	
0	33,000
0 (Transfusion with 500 cc. of Group IV citratd blood.)	
0	441,000
1	459,000
7	500,000
15	490,000
19	350,000
22	475,000
24	270,000
27	281,000
29	282,000
31	271,000
34	260,000
44	288,000
46	281,000
48	268,000
50	272,000
51	220,000
52	223,000
53	289,000
55	224,000
56	202,000
58	211,000
60	224,000
70	222,000
74	173,000
76	157,000
79	190,000
93	184,000
100	118,000
109	118,000

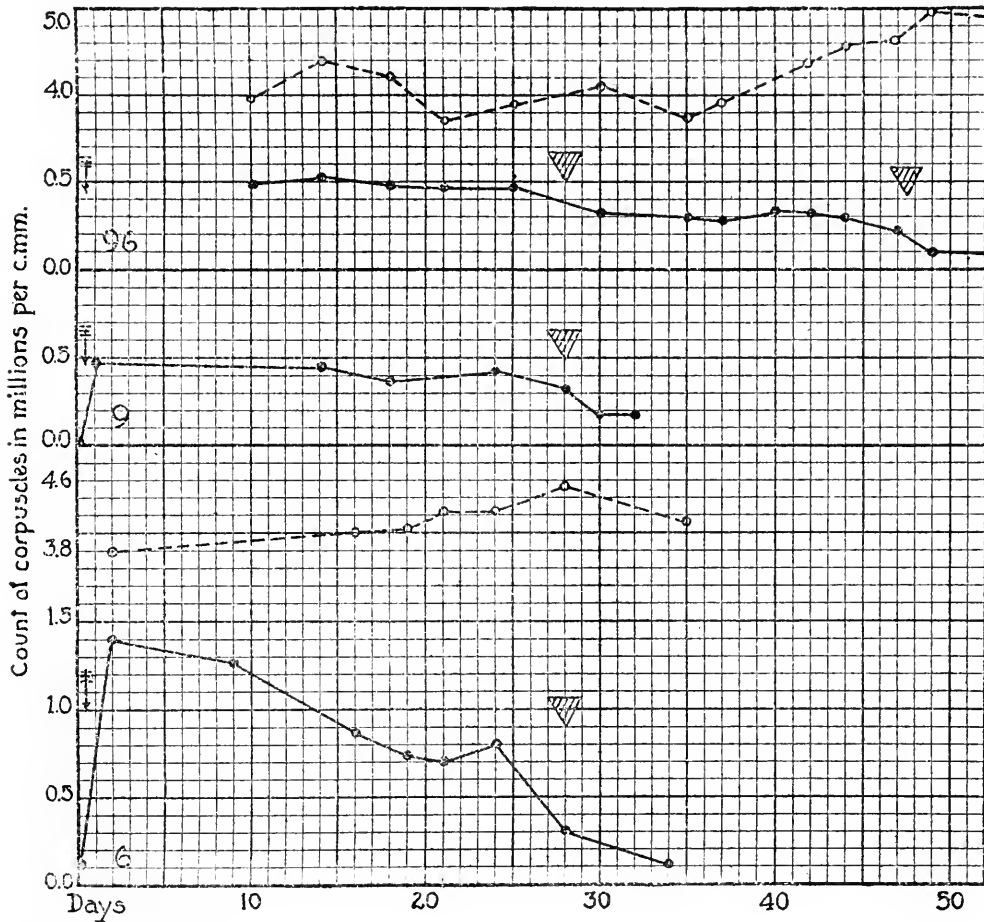
Text-fig. 4 presents the curves of Cases 6, 9, and 96, the protocols of which are given above.

Protocols of cases included in Text-fig. 5 are given below with the exception of Cases 48 and 115 which are presented above.

*Case 38.*—A woman, aged 32 years, weighing 133 pounds, in good general health had multiple lipomata, which were removed. The following day a transfusion of blood was given because of postoperative hemorrhage. Convalescence was excellent. Menstruation began on the 35th day after transfusion and ended on the 38th day.

*Case 47.*—A woman, aged 40 years, weighed 105 pounds. A partial thyroidectomy was performed, which was followed by a transfusion. The patient made an uneventful recovery. Menstruation began the 37th day after transfusion.

*Case 68.*—A woman, aged 36 years, weighing 82 pounds, had pernicious anemia. The patient claimed that her menstrual period was usually 24 days.

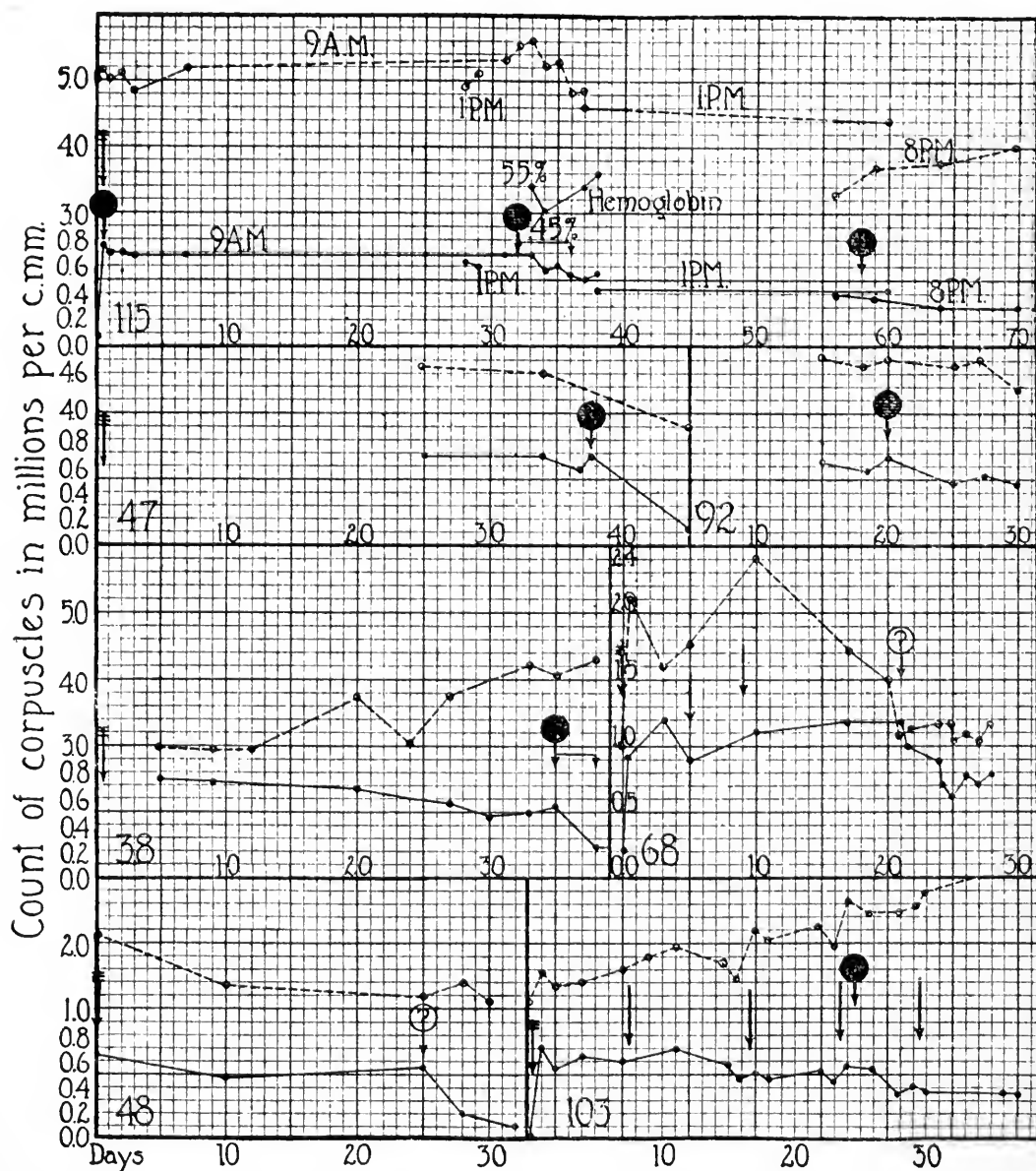


TEXT-FIG. 4. The drops in the curves of elimination of transfused blood of three women who received a transfusion with hysterectomy. The drops took place at approximately the same time after operation. — Unagglutinated corpuscles. - - - Total red blood count. ↓ indicates a Group IV transfusion. The cross-hatched triangle calls attention to periods of elimination.

While she was under observation menstruation occurred, beginning 3 days before transfusion, June 24, 1919. Menstruation did not occur July 18, when the patient expected it. The patient died August 24.

*Case 92.*—A woman, aged 49 years, weighing 215 pounds, had bleeding uterine fibroids, marked secondary anemia, and a degenerating colloid goiter. She was

given three transfusions, two of which were of Group IV blood, after which a partial thyroidectomy was done. Menstruation began 11 days after the last Group IV transfusion, but stopped immediately, although previously bleeding had been profuse.



TEXT-FIG. 5. Curves illustrating the drop in transfused blood that takes place at the menstrual period. ● indicates the beginning of a menstrual period; ↓ indicates the time at which menstruation should have occurred. — Unagglutinated corpuscles. - - - Total red blood count. ↑ indicates a Group IV transfusion; ↓ a like group transfusion.



*Case 103.*—A woman, aged 32 years, had pernicious anemia and received a series of transfusions. While under observation profuse menstruation took place, beginning the 24th day after the Group IV transfusion.

In examining these curves it must be borne in mind that several factors other than blood elimination will bring about changes in the corpuscle count. Of these the most important is due to dilution from increase in blood volume. Robertson and Bock have shown, and my experience corroborates theirs, that after hemorrhage there is often only a slow return to normal blood volume. It is probable, too, that changes in the capillary circulation cause changes in the blood count taken from the ear which do not represent changes in the average corpuscle content. Robertson and Bock have shown that in cases in which the blood volume was low the difference between the capillary and vein hemoglobin might be as much as 12 per cent of the vein hemoglobin. There seem also to be daily changes in the blood volume in certain patients. Curves are very much smoother in which the counts have all been made at the same time of day.

It will be seen in Text-fig. 2 that no definite elimination is shown in the curves there represented. In Case 95 there was no change that could be interpreted as an elimination of transfused blood in 51 days. In Case 115 after a slight initial drop, presumably from blood volume adjustment, the count was constant within errors of technique for 33 days. In this instance the counts were taken at the same time of day. In Case 93 the patient was under observation for 43 days, with no definite change other than the initial blood volume adjustment and adjustment after severe vomiting. In Case 97 there was no elimination in 51 days. As these curves maintain a level for a long time, indicating that during that time there was no elimination, it is obvious that, as the blood is eventually eliminated, it is not eliminated by a continuous process but by an intermittent process.

Text-fig. 3 shows two curves of elimination of transfused blood of men, one normal and the other with purpura of several years standing, but in fairly good health, and the third, of a woman beyond the menopause, who was jaundiced. It will be seen that in these curves there are periods of elimination which are short and probably would have proved to be shorter if the counts had been taken closer

together. The first drop in Case 119 took place between two counts spaced 2 days apart. The drop in Case 55 took place between counts that were taken 3 days apart. The first drop in Case 116 took place between two counts that were 5 days apart; the apparent further drop on the following day is due to a change in technicians. It would seem, then, that these drops take place rapidly.

It will be seen that there is a periodicity, although a somewhat irregular one, in the period of elimination of the two men, ranging in the normal man from 18 to 28 or 30 days. Accompanying these drops there tends to be an increase in the total blood count.

On examining the nature of the falls in the curves it will be seen that they are such as to suggest a blood-destroying activity on the part of the body rather than a spontaneous disintegration of simultaneously formed corpuscles. For if we postulate a blood destruction which is dependent solely on the condition of degeneracy of the corpuscle, we shall have to assume not only that blood formation takes place in short periods, which may be the case, although the continued presence of reticulated corpuscles would seem to indicate that there is at least some continuous formation, but also that the individual corpuscles are so uniform that the wear and tear which they experience after 3 months (Case 119) in the circulation bring them to a simultaneous disintegration point. But even if we are willing to make this assumption, further examination of the figures does not bear out this hypothesis. For if the condition of wear and tear were wholly responsible for initiating the destruction of the corpuscles, we should expect a regularity in the rate of their disappearance. If, for instance, the corpuscles which were transfused had been formed in sharp crises every 3 weeks and their life were 12 weeks, then one-fourth of the corpuscles was due to live 12 weeks, one-fourth 9 weeks, one-fourth 6 weeks, and so on. Equal amounts should have disappeared at each period of elimination. But this was not the case; by far the largest fall came in the first periods of elimination. In Case 119 the first drop was 200,000, or nearly one-half of the blood transfused, while subsequent drops were each approximately 80,000, or one-fifth of the original blood injected. In Case 116 the first drop was 150,000, or three-eighths of the blood injected, while subsequent drops were 90,000 and 80,000, about two-tenths

of the injected blood. These figures seem to place the elimination with reference to some active process on the part of the body, probably modified by the condition of the corpuscles and the number present in the circulation.

In Text-fig. 4 are included the curves of the three women studied who received Group IV transfusions immediately before or subsequent to hysterectomy. In each case a period of elimination came between the 25th and 30th days after hysterectomy. The elimination would probably have proved to be sharper if the counts had been taken closer together. In one of these women a second period of elimination occurred about 20 days after the first. This suggests, although the series is too short to have a conclusion drawn from it, that in some way the hysterectomy initiated a cycle equivalent to the menstrual cycle, and when complete elimination did not take place the subsequent periodicity of elimination was irregular as in the case of the two men.

Text-fig. 5 summarizes all the data that I have been able to obtain on the relationship between the elimination of transfused blood and the menstrual period. Six menstruations, and two periods when menstruation should have occurred but did not, were observed. There has been no exception to the fact that elimination of transfused blood that could not be accounted for by simple blood loss from the menstrual flow occurred during the menstrual period. It has not always happened that when menstruation should have occurred but did not that there was an elimination of transfused blood. In the two cases in which the elimination did occur without the appearance of the menstrual flow, the women were in a condition of extreme anemia, and it is probable that the suppression of the flow was due to the anemia rather than to the suspension of the menstrual process.

In Case 115, which was the most intensive study made, the patient's blood volume was low and her count showed a great variation during the day, being much higher in the morning than in the evening, so that only counts taken at the same time of day were comparable. Those taken through the menstrual period that came 32 days after transfusion were made in the morning by one observer; those during the menstrual period that came 58 days after transfusion were made

by a second observer, and in the evening. The count which determines the further end of the line connecting the two menstrual periods was made by the first observer at 1 p.m. The nearer end of the line is determined from a calculated figure. It will be noted that during the earlier menstrual period, although there was a decrease in the total red blood cell count, there was some increase in the hemoglobin. At this time the transfused blood had a higher index than the patient's blood, being 0.7 per cent, while the patient's blood was 0.5. During the later menstrual period there was a rise in the total blood count. In Case 38, with a very slight menstruation, there was an elimination of more than half the transfused blood; the preliminary fall in this curve is due to blood volume adjustment. There had been massive hemorrhage, and the patient's blood volume was low. In Case 103, that of a pernicious anemia patient, although the menstrual flow was profuse, there was a moderate fall in the transfused blood and a slight increase in the total count. In Case 48, in which menstruation did not occur when it was due, there was a complete elimination of the transfused blood with a slight temporary rise in the total blood count. In Case 68, one of pernicious anemia, a big fall in the Group IV transfused blood occurred beginning the 21st day, which may have been in part due to a blood volume improvement, since the patient's blood volume had been exceedingly low, and her general condition in the face of what seemed to be a period of blood loss became no worse. There was, however, at this time a marked elimination of the native corpuscles and of like group corpuscles, since the difference between the patient's whole blood count and the count of the transfused blood became so slight that it was within the limit of experimental error. From the 21st to the 25th days the difference between the two counts increased again, which in view of the practically complete elimination of the patient's own blood must indicate a coincident blood production.

#### DISCUSSION.

There seems to be little doubt that the elimination of Group IV transfused blood is brought about by an active destroying process of the body which is some part of a metabolic cycle, evidenced in

women by menstruation. Whether the Group IV transfused blood is eliminated as part of a general blood-eliminating effort of the body or whether it is eliminated as a foreign body is a matter of interest. The fact that the Group IV transfused blood stays in the circulation for considerable lengths of time would be an argument against its foreign relationship to the body, but, on the other hand, in my studies of Group IV transfusions in patients with pernicious anemia, the Group IV transfused blood has remained in the circulation in the presence of destruction of the patient's own blood, and in one instance, Case 68, in the presence of destruction of transfused blood of the same group as the patient's. It would seem that if the elimination of the Group IV blood takes place because of a normal blood-eliminating process, either there must be two processes, one of which can handle the blood of the unlike group, while the other cannot, or else that a certain pressure of elimination must be attained before the threshold is reached for the elimination of Group IV which is apparently more difficult to destroy. Unless it is assumed that some Group IV transfusions are, relative to the patient's own blood, less enduring than others, the latter would not seem to be the case because in some instances we have seen complete elimination of the transfused blood in one elimination period, which would, on the assumption of a higher threshold mechanism, necessitate tremendous coincident elimination of the recipient's blood. If we assume two processes at work in the physiologic elimination of blood, one of which is more able to handle the apparently slightly foreign Group IV blood, we might conceive of it as part of a more general lytic or phagocytic function which has as one of its expressions of activity the sloughing of the endometrium and which is able to overstep the group differences. On the other hand, this periodic elimination of transfused blood may not be due to any specific blood-eliminative activity, but to a non-specific eliminative activity.

It would be a matter of great interest to know whether or not normal physiologic blood elimination also takes place in sharp cyclic crises. Satisfactory evidence on this point is difficult to obtain because if there is such blood elimination it may be masked by production, or if there is any apparent drop in the total blood count we do not at present know that it is not due to changes in blood volume or to an uneven distribution of corpuscles.

Carnot and Deflandre, Pölzl, and Sfameni, who studied the changes in the red corpuscle count over the menstrual cycle, report an increase in the count before the menstrual period, with a fall upon the onset of menstruation, which fall has no relationship to the amount of blood lost in the menstrual flow. In anemic women an improvement in the blood was noted after menstruation. Carnot and Deflandre point out that the fall may be only apparent, due to the accumulation of corpuscles in the congested pelvic organs, but call attention to the various toxic phenomena which accompany menstruation and the vitality which follows the period. It is generally conceded that menstruation is not a local but a generalized process. The numerous instances of vicarious menstruation seem to place this point beyond discussion, and there is in addition supporting evidence derived from metabolic studies, such as creatine excretion, nitrogen retention, reported by Schrader, blood calcium reported by Bell, changes in blood cholesterol reported by Gonalons, and increase in basal metabolic rate reported by Ford. The fact that some generalized change takes place at this time and with it a destruction of transfused blood is strongly suggestive that the drop in the red count reported is also due to a corpuscle destruction process rather than to a change in blood volume or an uneven distribution of corpuscles. The fact, too, that the blood picture of anemic women has been observed definitely to improve after a menstruation, in addition to being evidence in favor of the crisis in corpuscle production, supports the idea of a coincident crisis of destruction.

#### SUMMARY AND CONCLUSIONS.

Group IV transfused blood in a recipient of unlike group is eliminated by a blood-destroying activity of the body.

This blood-destroying activity is periodic both in men and women, and in women coincident with menstruation.

The elimination of the transfused blood probably takes place as part of a period of blood-destroying and blood-producing activity of the body, although direct evidence to this effect is so far lacking.

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## STUDY OF TRANSFUSED BLOOD.

### II. BLOOD DESTRUCTION IN PERNICIOUS ANEMIA.

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Many of the older writers inclined to the idea that pernicious anemia is fundamentally a failure in erythrogenesis, but in the light of recent investigation it is more generally assumed that increased blood destruction is the important cause of the anemia seen in this disease. This assumption rests mainly upon increase in hemosiderin deposits in the tissues, increase in bile pigment output, the rapid falls that are seen in the blood count, and the anemia in the face of what is apparently a hyperplasia of the bone marrow. My own results of the study of thirty-three cases of pernicious anemia patients who received Group IV transfusions have not realized the intensive blood destruction that I expected to find.

Upon going over the literature of the subject I find it possible to put more than one interpretation upon these facts of increased bile pigment excretion, hemosiderin deposits, etc., which are usually assumed to be evidence of a high rate of blood destruction in pernicious anemia. The increased activity of the bone marrow as judged by the increased density of mitotic figures and immature cells and the increased mass of erythropoietic tissue may be only apparent. If there were some factor present which retarded the speed of the process of maturation and division, while the physiologic stimulus to erythrocyte production brought into play by the anemia caused an increase in the number of cells dividing, we might have such a picture with a decrease in the total cell output. The hemosiderin deposit is evidence only that iron is not being kept in circulation. This may be due to abnormal destruction of hemoglobin, but it is equally possible that it is due to a failure to utilize sufficiently quickly

in the formation of new hemoglobin, iron released by a normal rate of blood destruction. Increased bile pigment output cannot be accepted as evidence of increased blood destruction because Whipple and Hooper have shown that bile pigment is derived from other sources besides hemoglobin. It is conceivable that with a depression in protein synthesis the pigment material which might have combined with globin to form hemoglobin is diverted to the increase of the bile pigment output. Urobilin and urobilinogen in the duodenal content and urine, although they may appear in these excretions when there is blood destruction, are only evidence that bilirubin has been reduced before it reaches the intestinal tract or that it was incompletely synthesized. Rapid falls in the blood count can just as plausibly be explained on the basis of a failure of production and the physiologic end of the corpuscle as of its abnormal destruction. All these facts of hyperplastic bone marrow, increased bile pigment, etc., which from one angle seem to point collectively towards excessive blood destruction can, by changing the point of view, be made to present just as solid a front against it. I do not feel that our laboratory studies of pernicious anemia offer anything that can be accepted as absolute proof of excessive blood destruction.

Perhaps we have our best evidence of blood destruction in pernicious anemia when upon transfusion the total count fails to increase or even decreases and when the symptoms of the patient do not indicate any blood volume improvement. Since it is now believed that there is no intrinsic destructibility of transfused blood, even of citrated transfused blood, this failure of the corpuscle content to increase when corpuscles are injected is evidence of corpuscle destruction. This situation, however, is the occasional one and, unless the patient succumbs, is only temporary. It is evidence that there are at least intermittent periods of destruction, but it does not support the idea which some investigators have of a continuous destruction.

On the assumption of an increased blood destruction, the prevailing opinions as to its cause cover all the possibilities. Most of these can be included under three headings. We can conceive of an extraneous hemolytic toxin as causing injury to the blood corpuscles, resulting in their destruction, either by disintegration in the circulation or

by later elimination by the body. It is conceivable that a toxin is produced which does not act directly upon the blood cell in the circulation, but affects the bone marrow so that a type of corpuscle is produced which either becomes effete more rapidly and, in consequence disintegrates or is removed from the circulation, or which deviates from normal blood to such an extent that it is removed because of its foreign quality. Lastly, it is conceivable that without any injury to the corpuscles in the circulation or to corpuscle production, there might be a speeding up of the physiologic blood destruction agencies. It is also conceivable that all these effects might be produced not by a specific toxin, but by the toxic effect of some abnormal metabolic habit brought about by some past injury.

The most popular conception of the cause of blood destruction in pernicious anemia is the hemolytic toxin. Wells, in the new edition of his Chemical pathology, says: "Putting together the above findings, we see that in pernicious anemia we have every evidence that excessive hemolysis is taking place, and the fact that continued poisoning by toluylenediamine and other hemolytic poisons, such as that of *Bothriocephalus*, may give rise to a condition resembling pernicious anemia very closely, indicates strongly that hemolytic poisons are the cause of pernicious anemia."

Pappenheim says that other "hyperchrome" anemias, like pernicious anemias and hemolytic anemias, are at the same time hemotoxic, myelotoxic, and splenopathic, but for all these manifestations of the disease a single toxin, as in the case of toluylenediamine, may serve. He is convinced that in all cases the "hyperchrome" anemia is due to a toxin. He points out that these poisons, such as toluylenediamine and pyrodine, which produce the "hyperchrome" anemia, are not hemolytic. Pyrodine, for instance, does not always produce the "hyperchrome" anemia, but sometimes first kills the animal from general organ poisoning. When the "hyperchrome" anemia is produced, Pappenheim considers that it is due to a tendency of the animal organism to transform the pyrodine into hydroxylamine, which is a hemolytic substance. He thinks it probable that the various conditions, lues, purpura, pregnancy, *Bothriocephalus* infection, etc., which seem sometimes to cause pernicious anemia, do so because they establish some improper metabolic process, perhaps by injury to some unknown organ of internal secretion, so that the body forms the hemolytic toxin, hydroxylamine.

Bunting, who produced a condition resembling pernicious anemia with continued small doses of ricin, considers it probable that the disease is due to the long continued action of a hemolytic poison, which produces the blood picture by causing an overstimulation of the bone marrow. Hunter, Herter, and others found hemolytic substances in the intestinal tract and considered the anemia due to their absorption, but no experimental work establishes their view.

The production by Schauman and Tallquist of *Bothriocephalus* anemia in dogs with hemolytic extracts from the worm rests on too meager evidence to be accepted without further work. These investigators treated seven dogs with worm extract and pieces of worms and produced only slight changes in the blood in all but one, which died with a blood count of 3,400,000 and without typical changes in the blood picture. The claim is made that these dogs clinically showed symptoms of anemia, but no clinical symptoms typical of pernicious anemia are reported. Blanchard quotes Vlaiev's negative attempts to produce anemia in rabbits and pigeons with extracts of *Bothriocephalus* tapeworm. Seyderhelm, working with the bot-fly in horses, reports that he was able to reproduce the typical anemia, not with the extract, which proved to be hemolytic, but with an alcohol-insoluble material which *in vitro* has no solvent effect on red blood corpuscles.

Except for Bunting's ricin, which besides being hemolytic has other strongly toxic properties, the establishment of anything resembling pernicious anemia with a hemolytic toxin does not seem to have been clean-cut.

Among the men who incline to the idea that the blood destruction in pernicious anemia is due to incompatibility or low resistance of the corpuscles brought about by a toxin are Gulland and Goodall, and Hirschfeld. MacCarty, of this Clinic, considers it probable that the erythrogenic process has become malignant and that the corpuscles are eliminated rapidly from the circulation because of their foreign quality. We have no experimental proof of a lowered resistance of the corpuscle of pernicious anemia.

The possibility of an excessive activity of the physiologic blood-destructive process of the body is not emphasized in the literature, although mention is made of it. The idea that sepsis is the cause of pernicious anemia may be grounded in such a conception. Sajous points out that hemolysis occurs whenever toxins, either endogenous or exogenous, appear in the blood in quantities sufficient to excite a defensive reaction. The antibodies then attack not only the toxins, but the erythrocytes as well. Clark and Evans suggest the interesting possibility that the anemia of the so called hemolytic anemias may be due to a reduction in the power of the blood serum to protect the corpuscles from whatever hemolytic agencies they may come in contact with normally in the circulation and present experimental evidence that in these anemias there is a reduction of the protective power of the serum against sodium oleate.

The following attempt was made to obtain some understanding of the mechanism by which blood destruction is brought about, by means of the study of the elimination of Group IV transfused blood. The technique used was that already described. Thirty-three patients with pernicious anemia not of Group IV were given Group IV transfusions. Four of them were under observation for about 3 months, ten were observed for 1 month or more, eight for at least 17 days, and the rest for shorter periods of time.

The object of the study was first to answer the question as to whether or not there is any hemolytic toxin at work in pernicious anemia by comparing the curves of elimination of Group IV transfused blood in these cases with those obtained from patients without any blood disease. As a control, patients with other diseases, in which it was suspected that there might be increased blood destruction, were chosen for transfusion.

It was hoped that if no excessive destruction of Group IV blood resulted the study might produce data that would give some clue as to which of the other two possibilities, namely the intrinsic weakness of the corpuscle or the speeding up of normal blood destruction, is responsible for the anemia.

#### RESULTS.

Of the four pernicious anemia patients (Text-fig. 1) who were studied until the elimination of transfused blood was complete or almost complete, the time taken for complete elimination in two instances was 91 days (Case 43) and 100 days (Case 59) respectively. In another (Case 97) elimination was not complete in 100 days, and in the fourth (Case 57) there were still some transfused corpuscles after 83 days. These curves compare well with the longest complete curve of elimination obtained in a normal individual and are much longer than the other seven completed curves obtained for individuals without blood diseases (Ashby). Of these four patients, in one instance (Case 59) the transfusion brought about a remission which lasted for over a year. In the other three cases it was necessary to give repeated transfusions during the time that the patients were under observation.

*Case 43.*—The patient was a man, aged 49 years, weighing 163 pounds. He had no free hydrochloric acid in the stomach. He had glossitis and combined sclerosis of the cord. The red cell count was 1,900,000, with a hemoglobin of 44 per cent, and color index of 1.4+. The white count was 3,400, with 36 per cent lymphocytes. There was marked anisocytosis and moderate poikilocytosis. Diagnosis of pernicious anemia was made. A series of six 500 cc. transfusions of citrated blood was given.

*Case 57.*—The patient was a man, aged 63 years, weighing 157 pounds. He complained of a general increase in weakness, beginning 2 years ago, when he became sallow, lost his appetite, and his legs swelled. There were glossitis, and

numbness and tingling in the feet. The red blood count was 1,136,000, hemoglobin 25 per cent, color index 1.1+, leucocytes 4,200, polynuclear neutrophils 44 per cent, lymphocytes 56.5 per cent, normoblasts 3 per cent; marked anisocytosis and poikilocytosis. Diagnosis of pernicious anemia was made. A series of eight 500 cc. transfusions of citrated blood was given.



TEXT-FIG. 1. The curves of elimination of 500 cc. of Group IV transfused blood in four pernicious anemia patients, studied until elimination was complete or nearly complete.

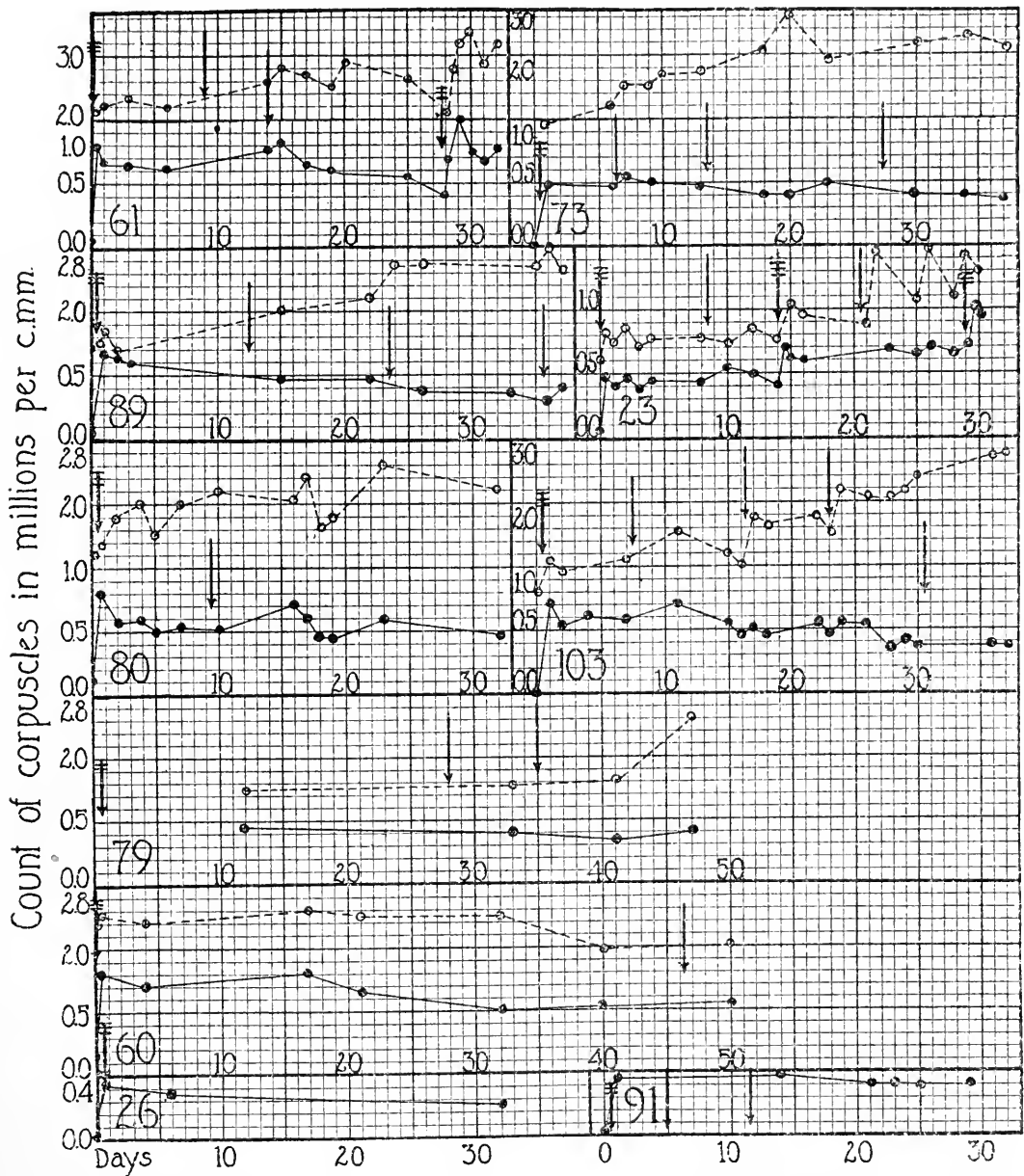
———— Unagglutinated corpuscles. - - - - Total red blood count. ‡ indicates a Group IV transfusion; ↓ a like group transfusion.

*Case 59.*—The patient was a short woman, aged 52 years, weighing 149 pounds. In February, 1918, when she first came up for examination, she was sallow and weak, had numbness of hands and feet, and a smooth tongue. Her red blood count was 1,900,000, color index 1.1+, leucocytes 3,400, and lymphocytes 72 per cent. There were moderate anisocytosis and poikilocytosis. Diagnosis of pernicious anemia was made. She was given 2,500 cc. of blood in three transfusions in the course of a month; this was followed 2 months later by two 500 cc. trans-



fusions. In May, 1919, she returned, with a red blood count of 1,330,000 and was given one 500 cc. Group IV transfusion, after which she improved and maintained a fairly good count to date.

*Case 97.*—The patient was a man, aged 42 years, weighing 155 pounds. His chief complaint was weakness. He had been gradually losing strength for 3



TEXT-FIG. 2. Elimination curves of Group IV transfused blood in ten pernicious anemia patients under observation for 30 days or more. — Unagglutinated corpuscles. - - - Total red blood count. ↓ indicates a Group IV transfusion; ↓ a like group transfusion.

years. He had glossitis, absence of free hydrochloric acid in the stomach, and tingling and numbness of the arms and legs. His red blood count was 2,240,000, hemoglobin 50 per cent, color index 1.1 —, and leucocyte count 4,000. The diagnosis made was pernicious anemia. The patient received a series of five (500 cc.) transfusions, of citrated blood. Later he returned for further transfusions.

*Case 23.*—The patient was a man, aged 57 years, weighing 139½ pounds. He suffered from weakness, dating back 7 years. 2 years previous to present date, with a blood count of 3,500,000 and a color index of 0.9+, glossitis, and posterior sclerosis, a diagnosis was made of pernicious anemia, of the neurotic type. A year later the patient returned, with a red blood count of 1,090,000, hemoglobin 26 per cent, color index 1+, leucocytes 3,800, and red cell count 830,000. A series of four 500 cc. transfusions of citrated blood was given.

*Case 26.*—The patient was a man, aged 44 years, weighing 148½ pounds. The skin was lemon-yellow, the tongue atrophic, and there was a systolic murmur over the base of the heart. He complained of weakness, which began 7 years ago. The hemoglobin was 32 per cent, red blood count 1,480,000, color index 1.1+, and leucocyte count 2,600. A diagnosis of pernicious anemia was made. The patient received a series of transfusions in 1918. He received transfusions in January, 1919, and again in May, 1919.

*Case 60.*—The patient was a man, aged 36 years, weighing 137 pounds. He was pale, and extremely weak. The neurological examination showed combined sclerosis. There was no glossitis. A test meal was not given. The blood picture was typical of pernicious anemia. A diagnosis of pernicious anemia was made. Three transfusions were given, with improvement in the patient's condition. There have been subsequent remissions.

*Case 61.*—The patient was a man, aged 42 years, weighing 152 pounds. There were weakness and combined sclerosis. The red cell count was 1,510,000, hemoglobin 34 per cent, color index 1.1+, leucocyte count 2,000. A diagnosis of pernicious anemia was made. Four 500 cc. transfusions of citrated blood were given.

*Case 73.*—The patient was a woman, aged 47 years, weighing 110 pounds. Her chief complaint was weakness. July 28, 1919. The red count was 1,750,000, hemoglobin 34 per cent, color index 0.9 —, leucocytes 5,000. There was marked anisocytosis. No test meal was given, but digestion improved with hydrochloric acid. Neurological examination showed spinal sclerosis of the pernicious anemia type. A diagnosis of pernicious anemia was made. The patient received a series of transfusions and returned home, where more transfusions were given. She died May, 1920.

*Case 79.*—The patient was a man, aged 25 years, weighing 126 pounds. He had a palpable spleen, and there was no free hydrochloric acid in the stomach. The neurological examination showed nothing, and there was no evidence of glossitis. His red cell count was 1,120,000, hemoglobin 23 per cent, color index 1,



leucocyte count 4,800, neutrophilic myelocytes 4.5 per cent, normoblast 4 in a count of 200, and megaloblasts 1. Diagnosis of pernicious anemia was made. Three 500 cc. transfusions of citrated blood were given. Since then the patient has returned twice for transfusions.

*Case 80.*—The patient was a man, aged 34 years, weighing 154 pounds. He had a bald tongue, but no evidence of combined sclerosis. His chief complaint was weakness and indigestion. The red cell count was 1,960,000, hemoglobin 40 per cent, color index 1+, leucocyte count 6,400, with small lymphocytes 48 per cent. There were moderate anisocytosis and poikilocytosis. Diagnosis of pernicious anemia was made. (On the 15th day after transfusion the patient took magnesium sulfate for stool examination.) Two transfusions of 500 cc. of citrated blood were given.

*Case 89.*—The patient was a woman, aged 60 years, who normally weighed 180 pounds and at time of examination weighed 127 pounds. Her chief complaint was weakness. Transfusions elsewhere had improved her condition. She had pigmented skin, sore tongue, and no free acid in the stomach. The red blood count was 1,820,000, hemoglobin 38 per cent, and color index 1. The leucocytes were 6,200, lymphocytes 33.5 per cent, and normoblasts 1 per cent. There were marked anisocytosis and poikilocytosis. Diagnosis of pernicious anemia was made. A series of four transfusions of citrated blood (500 cc.) was given.

*Case 91.*—The patient was a woman, aged 53 years, weighing 137 pounds. She showed marked weakness, glossitis, dyspnea, short pulmonic systolic murmur, edema, and early symptoms of combined sclerosis. Her color was lemon-yellow. Red blood cell count was 1,440,000, hemoglobin 35 per cent, color index 1.24, leucocytes 6,600, with polynuclear neutrophils 56 per cent, small lymphocytes 39.5 per cent, large lymphocytes 2.5 per cent, eosinophils 1.5 per cent, neutrophilic myelocytes 0.5 per cent, normoblasts 1.5 per cent, megaloblasts 0.5 per cent; moderate anisocytosis and poikilocytosis. The diagnosis was pernicious anemia. Three transfusions (500 cc.) of citrated blood were given.

*Case 103.*—The patient was a woman, aged 52 years. She complained of weakness. She was well until a year before, when she had influenza. There were glossitis, posterolateral sclerosis, soft systolic murmur at the apex of the heart transmitted to the axilla, and retinitis of pernicious anemia. The spleen was palpable 1 inch. The red cell count was 960,000, hemoglobin 23 per cent, color index 1.2+, leucocytes 2,400, polynuclear neutrophils 13.5 per cent, small lymphocytes 78 per cent, large lymphocytes 8 per cent, neutrophilic myelocytes 0.5 per cent, normoblasts 12 per cent, megaloblasts 1 per cent; marked anisocytosis and poikilocytosis. While under observation profuse menstruation took place, beginning December 20, 1919. A series of four transfusions of citrated blood (500 cc.) was given.

In the curves in Text-fig. 2 it will be seen that in no case is there any elimination of Group IV transfused blood that cannot be du-

plicated in the control cases with no blood disease (Ashby), and the majority do not show any drop as great as that which occurred in normal individuals. In Case 89 the fall up to the 22nd day would seem to be blood volume improvement, as the patient's blood volume, calculated from the transfusion data immediately after transfusion, was low, being 6.4 per cent of the body weight, whereas the blood volume in secondary anemia as found by this method is 8 to 9 per cent of the body weight. The same may be said of the apparent fall in Case 60. In Case 26 the count of unagglutinated corpuscles with a blood volume of 8.5 per cent would have been 342,000, so the count of this patient on the 6th day after transfusion was still more than the number that would have been present if a normal blood volume adjustment had been attained. As there was no count taken between the 6th and 32nd days, the patient having gone home in the meantime, we have no data as to when the drop which occurred in the curve between those dates took place. We have no reason, however, to assume that it was any sooner than would have been normal.

Group IV blood transfusions in seven cases of pernicious anemia were studied over periods varying from 15 to 26 days. These protocols have not been included to save space. In a case studied for 28 days two Group IV transfusions were given during that time. At the end of the period there were 934,000 Group IV corpuscles per c.mm. in the circulation. The initial Group IV transfusion had given 488,000 Group IV corpuscles per c.mm. The count of 934,000 would seem to account for two transfusions. In a case studied for 24 days the count of unagglutinable corpuscles fluctuated between 738,000 and 596,000 for the first 3 days after transfusion. After 24 days, the count was 627,000. A case studied for 20 days showed an initial count of 509,000 Group IV corpuscles and a final count of 507,000. Two cases studied for 19 days showed in one instance no elimination, as the initial Group IV corpuscle count was 436,000 and the final, 428,000. In the other instance there may have been some elimination between the 17th and 18th days, as the count changed from 440,000 to 389,000, although judging from previous fluctuations and the fact that the condition of the patient markedly improved although the total red blood count also dropped

somewhat it would seem possible that this drop was due to a blood volume increase. A case studied for 15 days gave an unagglutinated count of 548,000 on the 2nd day after transfusion and 542,000 on the 15th day. In none of these cases does there appear to have been any elimination of Group IV transfused blood during the periods studied, except perhaps in the case that was studied for 18 days, and here the decrease in count, if it was due to elimination and not to blood volume change, can be duplicated in a normal curve. Eight other cases studied for shorter lengths of time showed no elimination of the Group IV transfused blood.

In contrast to the stability of transfused blood in pernicious anemia cases is the elimination which was found to take place in certain patients with other conditions. In the cases which came under observation during the course of this study five appear to have shown a progressive and rapid elimination of Group IV transfused blood. This feature was most markedly shown in Case 118, one of hemolytic jaundice, in which there was a continuous fall in the curve of transfused blood until splenectomy, when the fall ceased sharply. Two cases of aplastic anemia (Cases 81 and 102, Text-fig. 3) one of myelogenous leucemia (Case 39), and one of carcinoma of the kidney with metastasis show rapid elimination of the transfused blood. The protocols and chart follow.

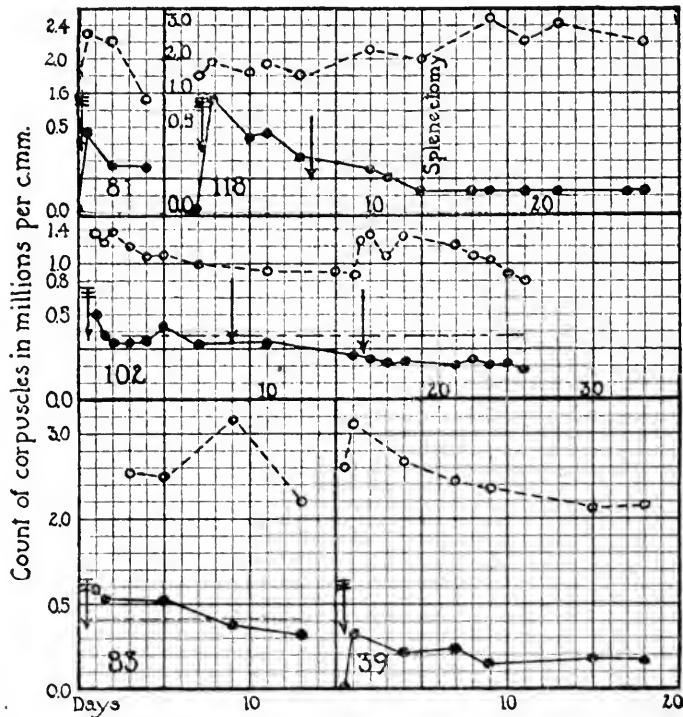
*Case 39.*—The patient was a man, aged 55 years, weighing 166 pounds. He had myelogenous leucemia with a white blood count of 215,000. A transfusion was given, followed after 6 days by a radium treatment. Profuse sweating occurred the 15th day after transfusion, with edema of the eyes and forehead; on the 18th day the edema was extreme. The patient died on the 19th day.

*Case 81.*—A man, aged 45 years, weighing 154 pounds. Diagnosis of aplastic anemia was made. The patient died August 29, 1919, of bilateral bronchopneumonia on the 4th day after transfusion. There were miliary tubercles in the spleen.

*Case 83.*—The patient was a man, aged 69 years, weighing 105 pounds. There was carcinoma of the left kidney, with metastasis. A left nephrectomy was followed by a transfusion with 500 cc. of Group IV citrated blood.

*Case 102.*—The patient was a man, aged 28 years, weighing 124 pounds. In October, 1918, he had influenza, which was followed by weakness and bleeding. There was severe epistaxis in October, 1919. Upon examination in November, the red blood count was 1,160,000, and the hemoglobin 13 per cent, with a color index of 0.5+. The leucocytes were 10,800, with polynuclear neutrophils 25

per cent, small lymphocytes 37.5 per cent, large lymphocytes 35.5 per cent, eosinophils 0.5 per cent, basophils 0.5 per cent, and neutrophilic myelocytes 1 per cent. There was moderate anisocytosis, and the platelet count was 134,000. December 9. The platelet count was 96,000. A diagnosis of aplastic anemia was made. Four transfusions were given. December 12. An eruption appeared upon the arms and face, which proved to be smallpox. January 1, 1920. The patient died.



TEXT-FIG. 3. Control cases in which there appears to have been some progressive elimination of Group IV transfused blood. — Unagglutinated corpuscles. - - - Total red blood count. - - - - The count of unagglutinable corpuscles that would be expected in an individual the size of a patient with a blood volume of 8.8 per cent of the body weight. ↓ indicates a Group IV transfusion; ↓ a like group transfusion.

*Case 116.*—The patient was a boy, aged 13 years, weighing  $76\frac{1}{2}$  pounds. He was jaundiced and weak. He had an enlarged spleen, which had been first noticed 3 months previously. The red blood count was 1,750,000, and hemoglobin 30 per cent, giving a color index of 0.8+. The white cell count was 19,500, with 65 per cent polynuclears and 25 per cent lymphocytes. The platelets were 148,000, and the bleeding time  $\frac{1}{2}$  minute. There was increased fragility. The blood picture did not improve after two transfusions. There was no evidence of familial jaundice. A diagnosis of hemolytic jaundice was made. Splenectomy was performed and the patient regained normal health.

When the curves of the total red blood counts of the pernicious anemia patients are examined, it will be seen that, although in most cases it would be difficult to say that there has been blood destruction during the preceding transfusion studies, there are some instances in which blood destruction is definite. In estimating these curves it must be borne in mind that changes in the blood volume very materially influence the total count. Changes in the number of transfused blood corpuscles per cubic millimeter give some indication of changes in blood volume. When the transfused blood count rises it is probable that the blood volume decreases, and when the transfused blood count falls with a subsequent return, showing that the fall was not due to elimination, it is probable that the blood volume increases. This has been assumed in the following calculations.

In Case 23 on the 10th day after the initial Group IV transfusion the total count was 1,100,000, the Group IV transfused blood was 520,000 minus the initial unagglutination of 70,000, or 450,000. Since the subsequent Group II transfusion 2 days previously was 100 cc. larger than the Group IV transfusion, it should account for at least the same number of corpuscles. The total count before this transfusion had been 1,170,000 with apparently a larger blood volume, as the count of the unagglutinable corpuscles was less. There have been added at least 450,000 corpuscles per c.mm., the blood volume has decreased, which should have still further increased the total red cell count, but the total count is still what it was before transfusion. We have here evidence of destruction. In Case 61 on the 15th day after the Group IV transfusion the total count was 2,250,000. The Group IV corpuscles were 700,000, so the two like group transfusions which had been given on the 9th and 14th days should have added 1,400,000, or twice that amount. The total count 3 days before these transfusions was 1,570,000, when the blood volume, judging from the unagglutinable corpuscle count, was somewhat higher; 1,400,000 corpuscles per c.mm. have been added to the blood, the blood volume has decreased, which would still further increase the count, and the total count has increased only 680,000. Here there is a loss of over 700,000 corpuscles per c.mm. within 8 days. In Case 73 on the 20th day after the Group IV

transfusion the total count was 3,000,000 and on the 23rd day it was 2,300,000, with a decrease in blood volume, giving a loss of more than 700,000 corpuscles per c.mm. In Case 80 between the 10th and 16th days after the Group IV transfusion, during which time a like group transfusion was given, there was a decrease of 150,000, with a decrease in blood volume, following purgatives, of nearly one-third. Here the loss was over 800,000 cells per c.mm. In Case 29, at the time of the like transfusion given February 10, the total count was lower afterwards than before, although the unagglutinated counts showed the volume to be about the same. In a case studied for 26 days there was a fall of 600,000 in the blood count within 3 days with no volume increase to account for it.

Of course, it must be admitted that in these estimates we are omitting the consideration of one factor; we do not know what is happening on the blood production side. The destruction may be greater than it appears to be. The periods of destruction, however, are limited to certain parts of the curve. The blood destruction is by no means of the same order as that seen in the case of hemolytic jaundice studied. It is quite possible that it is of no greater magnitude than that which is probably connected with the cyclic elimination of transfused blood (Ashby) which has been seen to take place in normal individuals.

There are, however, two cases in which blood destruction seems to have been excessive. These cases are of especial interest because the blood destruction of the corpuscles of the like group was so great that practically nothing was left but Group IV transfused blood; Group II transfused corpuscles were eliminated with the patient's corpuscles.

In Case 57 (already given) on August 21 the count of unagglutinable corpuscles after two Group IV transfusions, which had been made subsequent to a Group II transfusion, was 940,000 per c.mm. The initial unagglutinated count was 10,000 per c.mm., making the Group IV corpuscles 930,000. The total count was 1,230,000, making a difference of 300,000 to account for the patient's own blood and a Group II transfusion given 16 days previously, which would at the time probably have given a count of 450,000 per c.mm. On the following day the count of unagglutinable corpuscles was

still 940,000, while the total blood count was 930,000, or, within experimental error, the same. Unfortunately, however, this count was not checked by a duplicate tube as one of the tubes leaked in transit and a transfusion was given before we could get another count.

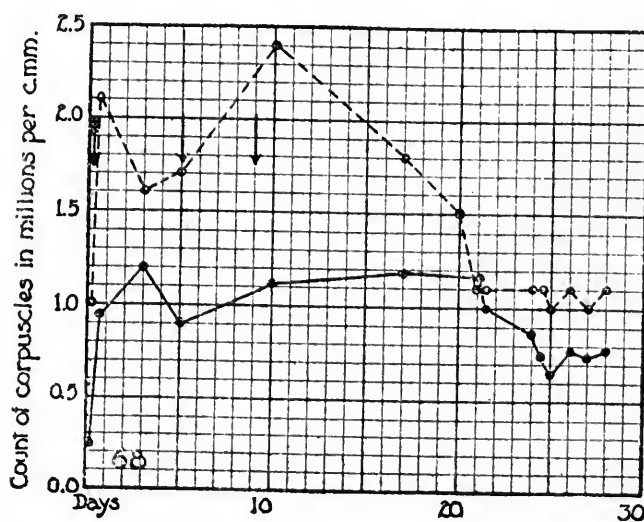
In Case 68 (Text-fig. 4), a woman, aged 36 years, who had had an excellent remission following splenectomy, received a Group IV transfusion on June 27. The patient's blood volume at the time was low, as the 500 cc. transfusion gave a count of 930,000 unagglutinated corpuscles per c. mm. On the 5th and 9th days after the Group IV transfusion she received 500 cc. transfusions of Group II citrated blood. The day after the second like group transfusion the count of unagglutinated corpuscles was 1,120,000 per c. mm. Since the initial count of unagglutinable corpuscles was 230,000 (this is unusually high) we take 890,000 as the count of Group IV corpuscles. The two Group II transfusions with the same blood volume should have given 1,780,000 corpuscles per c. mm. The total count minus the Group IV count was 1,610,000. On the 17th day after the Group IV transfusion the Group IV transfused corpuscles were 950,000 per c. mm.; the Group II transfused corpuscles should have been at least 1,900,000 per c. mm., or twice the number of Group IV corpuscles. The total number of Group II corpuscles, including both the patient's own corpuscles and the transfused, was 850,000. On the 21st day the total blood count and the count of the unagglutinable corpuscles were equal within experimental error. There were practically no clumps in the field, and a large number of nucleated red cells in the film. The potency of the serum was checked. A count repeated in the afternoon gave 990,000 for the unagglutinable corpuscles, 760,000 for the Group IV corpuscles, and 1,100,000 for the total count. This would have left 340,000 Group II corpuscles per c. mm. Since the count of the transfused Group II corpuscles alone should have equaled 1,500,000 per c. mm., we have here undoubtedly an elimination of the Group II transfused corpuscles as well as the elimination of the patient's corpuscles.

*Case 68.*—The patient was a woman, aged 36 years, weighing 82 pounds. She was very weak. Elsewhere a diagnosis of pernicious anemia had been made, and splenectomy performed April 28, 1917, after which there had been very great improvement in the patient's condition. On June 24, 1919, the red blood cell



count was 1,060,000, hemoglobin 28 per cent, color index 1.3, leucocyte count 7,400, lymphocytes 74.5 per cent. In counting 200 white cells, 89 normoblasts and 3 megaloblasts were seen. There were marked anisocytosis and poikilocytosis. Menstruation was expected on the 21st day after the transfusion but did not occur. The patient died a month later.

We have two cases (Nos. 64 and 49) which appear to be exceptions to the rule that in the pernicious anemia patients so far studied there is no increased rate of elimination of Group IV transfused blood. In Case 64 when the total red blood count was 1,400,000 a 500 cc. Group IV transfusion was given. The



TEXT-FIG. 4. Curves of a patient who showed elimination of the Group II transfused corpuscles as well as elimination of her own corpuscles. — Unagglutinated corpuscles. - - - - - Total red blood count. ‡ indicates a Group IV transfusion; ↓ a like group transfusion.

next day the count of unagglutinable corpuscles was 600,000. 5 days later when a second count was made the unagglutinable corpuscles had dropped to 99,000, which meant practically complete elimination of the transfused blood. The patient's total count was then 1,100,000. A Group II transfusion was given and 4 days later the total red blood count was 700,000. The patient died within a month. In Case 49 with the patient's initial blood count at 1,100,000 a 500 cc. Group IV transfusion was given, which gave 620,000 unagglutinated corpuscles per c. mm. and raised the total count to 1,500,000. The next day the unagglutinated count was 170,000



while the total count was 1,200,000. On the following day the unagglutinated count fell to 50,000, which was practically complete elimination, with a return in the total count to 1,100,000, the level before transfusion. There was no hematuria or transfusion reaction. The transfusion blood apparently was eliminated within 2 days, while the patient's own blood was unaffected. The only objection to accepting these data is that at the time the work was done I did not realize the possibility of a serum becoming non-specifically agglutinating and I did not check the Group IV serum used with this point in view. When in a third case this same elimination appeared to be taking place it proved to be the fault of the serum. On going back over the work I find that in each case on one of the days on which these counts were made, counts were made on other patients which gave the expected number of corpuscles, but I am not sure that the same serum was used. Since I have checked this factor it is true that I have not had a case in which rapid elimination of Group IV corpuscles occurred, but it is also true that with one exception I have not had an opportunity to study patients who were so near the terminal stage.

*Case 49.*—The patient was a man, aged 68 years. He had had previous relapses. There was no free hydrochloric acid in the stomach. The red blood count was 1,930,000, hemoglobin 40 per cent, giving a color index of 1. The white blood count was 2,400. The diagnosis was pernicious anemia.

*Case 64.*—The patient was a woman, aged 36 years. She was very anemic and thin. For 18 months she had been growing gradually weaker, and was in an exhausted condition at the time of examination. There was evidence of combined sclerosis. The hemoglobin was 22 per cent, the red blood count 1,040,000, color index 1.0+, and leucocyte count 1,900. The diagnosis was pernicious anemia.

#### DISCUSSION.

This study of pernicious anemia patients during a series of transfusions seems to indicate that although in some cases at certain times there is much blood elimination, on the whole, blood destruction is quiescent. Of course, it must be recognized that in this estimate the factor of production is an unknown quantity. Group IV transfused corpuscles are, as a rule, not eliminated so quickly as they are in transfused persons without blood disease, so that so far as Group

IV corpuscles are concerned there is no abnormal blood elimination. The fact that Group IV corpuscles are not eliminated rules out the activity of any corpuscle poison during these periods. For in order to assume the activity of such a poison we will have to make it one that is not able to attack Group IV corpuscles, in which case the incidence of pernicious anemia should at least be lower among Group IV individuals than among others. In the 189 pernicious anemia patients that have presented themselves at this Clinic during this study 52 per cent were in Group IV, which happens to be a somewhat higher incidence than the occurrence of Group IV individuals found in general among our patients and donors. It must be that at the time when the patients were under observation there was no hemotoxin active. It has been argued that transfusion introduces something which neutralizes the hemotoxin. It does not seem probable that there would be a complete neutralization of the toxin and that the patient's symptoms would still persist. Although in some instances a remission was brought about by the transfusion, in most cases the patients returned for further transfusions after 3 months. 3 months, it will be remembered, is the average length of time that what were presumably the youngest Group IV transfused corpuscles lasted in four pernicious anemia patients who were under observation to the end of elimination, and it is probably also the length of life of the like group transfusions. As in a few instances Group IV transfused blood stayed while the patient's total blood count dropped greatly, it would not seem that the protection of the Group IV corpuscles was due to an antibody to this hypothetical hemotoxin introduced by the transfused blood.

In the two cases, Nos. 75 and 68, in which there was the most marked elimination, the Group IV transfusion was maintained and both the patients' corpuscles and the transfused like group corpuscles were eliminated. Since normal like corpuscles were eliminated, the elimination in these cases cannot be assumed to be due to any intrinsic weakness of the corpuscle. Since in this elimination there was a differentiation between like group corpuscles and Group IV corpuscles it would seem that the factor bringing it about must have been the result of an activity of the body itself. If we accept the two cases in which the transfused blood disappeared rapidly.

we have additional data that would seem to bear out this conclusion. In the case in which there were time data, the normal transfused blood disappeared in 2 days, while the count of the patient's blood was maintained. Here again there would seem to be a differentiation between groups which would seem to be attributable only to a physiologic blood-destroying function.

This speeding up of the blood-destroying activity of the body, which seems to be the basis of the abnormal periods of blood destruction which are seen in transfused pernicious anemia patients, might be due, it is conceivable, to the indirect effect of a present toxin, or it might be due to some past injury which makes it difficult for the organism to maintain a sufficient supply of some substance necessary to colloidal equilibrium of the corpuscles. The recent work of Clark and Evans showing a reduction in the hemolytic inhibitory powers of serum of pernicious anemia patients to a point markedly below what is found in normal serum, and my own finding that at least as far as transfused blood is concerned physiologic elimination is brought about by a spasmodic effort on the part of the organism, are suggestive with regard to the mechanism by which these sudden excessive eliminations seen in pernicious anemia are brought about.

#### SUMMARY AND CONCLUSIONS.

Evidence is presented to show that there is no hemolytic toxin producing the anemia in pernicious anemia.

Partial evidence is presented to show that the periods of active blood destruction which are seen as the exception in pernicious anemia cases during a series of transfusions are due to the activity of the blood-destroying organs of the body rather than to the intrinsic weakness of the pernicious anemia blood corpuscle.

It is questionable whether blood destruction is as important a factor in producing the anemia of pernicious anemia as it is at present usually assumed to be.

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## EXPERIMENTAL SYPHILIS IN THE RABBIT.

### VII. AFFECTIONS OF THE EYES.

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PLATES 7 TO 9.

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Infection of the eyes following inoculation of the genitalia was one of the first forms of generalized syphilis to be recognized in the rabbit and has been reported more often than any other metastatic condition. Our knowledge of these affections dates back to 1908-10 when the first cases of metastatic keratitis were reported by Grouven (1-3), Mezincescu (4), Uhlenhuth and Mulzer (5), and Truffi (6). Since that time, several forms of affections have been described. Those most frequently mentioned are conjunctivitis, pericorneal injection, and interstitial keratitis; of these, keratitis is perhaps the only one which possesses a clearly defined status.

In addition to this class of affections, lesions of the eye grounds including choroiditis and chorioretinitis were described by Nichols (7) and by Reasoner (8) working in collaboration with Green. With certain strains of *Treponema pallidum*, these conditions were noted in from 70 to 95 per cent of the animals examined (mode of inoculation not stated) which may indicate that lesions of the eye grounds are more common than those of other structures—at least with some strains of the organism.

Reasoner also reported an instance of cataract of both eyes and a gumma involving the ciliary body, the iris, and the lens.

Our own observations on affections of the eyes were confined to a study of those conditions that could be detected by the unaided eye and included, therefore, affections of only the more superficial structures such as the conjunctiva, the cornea, and the iris. This group of lesions has received more attention than any other class of syphilitic affections but there is still much which may be added to the facts

already recorded. In presenting the results of our observations, we shall not attempt to do more than describe changes which might be recognized by any one familiar with the simpler forms of eye lesions.

The difficulties of their sharp delimitation will doubtless be appreciated, and while the various groups of affections are separated from one another, as a matter of convenience in presentation, it must be understood that combination was one of the most characteristic features of eye involvement.

### *Pericorneal Injection.*

Pericorneal or ciliary injection is a well recognized condition in rabbits infected with *Treponema pallidum* but in the literature it is practically always referred to in connection with keratitis and apparently no significance has been attached to it apart from this one condition. It is, however, the most frequent sign of infection of the eyes and was often noted in animals where no other gross abnormality could be detected as well as in association with affections of the conjunctiva, the cornea, and the iris.

It occurred in two fairly distinct forms. The one most often seen consisted of a ribbon-shaped band of distended vessels which extended from the fornix to the sclerocorneal margin (Fig. 1) where they spread out and partially encircled the cornea. The usual position of these vessels was the region of the superior rectus muscle but they were also seen in the lower or lateral sectors of the eye.

A second type of pericorneal injection, which is perhaps more analogous to that seen in the human eye, consisted of a series of prominent vessels which encircled the cornea, giving off a few small collaterals which radiated outward as shown in Figs. 2 and 3.

One of these forms appeared to be as characteristic as the other, and in the most pronounced cases, the two were combined in about equal proportion with zonal radiations extending outward from all parts of the cornea. This was the state of the eye in Fig. 1 which was a marked case of pericorneal injection associated with a moderate degree of acute conjunctivitis (shown on the upper lid), but as may be seen, there was no involvement of either the cornea or the iris. This condition lasted for only about 48 hours when the congestion subsided and the eye returned to normal.

Ciliary injection was the first sign of eye infection to appear and the history of a simple injection was usually that of a recurrent affection of short duration. During its early stages and in mild cases, it suggested no more than a vasomotor instability of some particular group of vessels which would suddenly become engorged and return to normal within a very short time, leaving some uncertainty as to whether any abnormality existed. Then a well established injection would take place and subside after a few days or the condition would gradually develop into an outspoken iritis or keratitis.

As yet, no attempt has been made to demonstrate spirochetes in a case of simple ciliary injection. Histologically, however, there were definite pathological changes. These consisted of vascular dilatation and engorgement of the vessels in the episcleral tissue and at the sclerocorneal junction; there were slight edema of these tissues and a moderate polyblastic infiltration which was chiefly perivascular. The identification of this affection as a manifestation of syphilis rests, therefore, upon clinical and histological evidence. A marked ciliary injection presents no difficulties of diagnosis, but unless the milder cases are followed by some more characteristic lesion, they cannot be recognized clinically as more than probable manifestations of syphilitic infection. The significant features are its transient character, the tendency to recurrent attacks, the frequency with which it eventually terminates in iritis or keratitis, and its constant association with these two conditions.

### *Conjunctivitis.*

Inflammation of the conjunctiva was noted in connection with affections of both the nasolacrimal system and of the eye itself. In the latter instance, it usually preceded or was an accompaniment of other affections of the eye, and it was difficult to determine clinically whether the inflammation of the conjunctiva was the result of a conjunctival infection or only an associated manifestation of an infection centered elsewhere. There were instances, however, in which the conjunctiva appeared to be the chief or sole focus of infection, and, in any case, involvement of the eyes frequently gave rise to marked inflammatory reactions which might affect the entire conjunctiva or only certain limited areas.

An acute diffuse inflammatory reaction was frequently seen in association with a simple ciliary injection or during the early stages of a keratitis or iritis. This form of affection began with reddening and swelling of the margins of the lids and conjunctiva, increased lacrimation, and drooping of the upper lids (Figs. 3, 11, 12, 17, and 19). It progressed rapidly and in some instances resulted in an intense congestion and swelling of the entire membrane with petechial hemorrhages and gray or yellowish gray patches scattered here and there.

In many instances, the inflammatory reaction was confined to a smaller area, the usual seat of such processes being the region of the superior rectus muscle or some part of the palpebral conjunctiva.

The acute manifestations were, as a rule, of short duration and disappeared completely within a few days.

No spirochetes were found in the lacrimal secretion of animals with this type of inflammatory reaction, and no effort was made to demonstrate them in the conjunctiva itself, but sections of the conjunctiva showed a characteristic syphilitic infiltration.

Another form of conjunctival lesion which was frequently seen in rabbits infected with *Treponema pallidum* resembles somewhat the phlyctenular conjunctivitis of man. These lesions were of three types, not all of which could be related to the syphilitic infection. In one group of cases, they appeared as distinct nodules or thickened patches situated at the sclerocorneal margin as in Figs. 4 and 5 or distributed along the course of the vessels in the superior quadrant of the eye (Fig. 6). The nodules were of a pale, opalescent, or yellowish white color and were associated with a well marked ciliary injection as in Fig. 4 or with a vascular reaction which was confined to the affected part of the eye as in Fig. 5.

Usually, the nodules or patches remained comparatively small but in one instance a lesion of this type developed into a mass which practically filled the superior fornix and presented all of the characteristics of a syphilitic granuloma, as may be seen by reference to Fig. 6.

Dark-field examination of the lesions for spirochetes gave inconsistent results. Some of them showed spirochetes in abundance, while in others, no organisms were found. The diagnosis of this group of



conditions was not difficult, however, since they were nearly always followed by or associated with characteristic lesions of the cornea or iris.

A second group of phlyctenular lesions differed from those described chiefly in that they were composed of small, ill defined, gelatinous masses of a pale pink or salmon color. Some of them presented much the same appearance as that in Fig. 5, while others showed no injection of the conjunctiva or episcleral tissues. An example of a lesion of this type is given in Fig. 7.

These affections were at times comparatively common in both infected and uninfected animals. No spirochetes have been found in them, but in some cases, the condition has progressed to a definite keratitis. In the absence of such a history, we know of no clinical means by which the etiology of lesions of this type can be determined.

There is also a third condition, analogous to those just described, which may be referred to briefly. The appearance presented is shown in Fig. 8. There were a series of small discrete nodules of a slightly translucent or opaque white color distributed along the sclerocorneal margin; there was no increased vascularity of the area, and in the few cases seen, the nodules remained essentially as they appear in this photograph. No spirochetes have been demonstrated in lesions of this type, and there is no evidence, either clinical or pathological, to indicate that they are of syphilitic origin, unless it be the fact that they are composed of masses of lymphoid cells which occupy the same position as analogous lesions of established syphilitic origin.

#### *Keratitis.*

Keratitis is the most easily recognized lesion of the eye and (with the possible exception of metastatic lesions of the testicle) has been reported more often than any other manifestation of a generalized infection. It might appear from this that keratitis is the most common of the generalized lesions but such is not necessarily the case; it may be either very common or very rare, depending upon a number of circumstances. As the infection is ordinarily propagated, however, keratitis is of very frequent occurrence with most strains of *Treponema pallidum*.

The form of keratitis usually observed in the rabbit is always preceded by a ciliary injection and frequently by a diffuse inflammatory reaction of the conjunctiva. The lesion described by all writers is an interstitial or parenchymatous keratitis. It is peripheral in origin, and while it may develop from any part of the cornea, or from its entire circumference, the most common location is the superior margin.

The corneal lesions appear either in the form of a delicate fringe of vessels which extend over the edge of the cornea or as a narrow zone of turbidity. As the vascular network spreads, the cornea becomes clouded, or as the zone of infiltration extends centrally, it is followed by the development of a network of vessels so that in either case the lesion produced combines the two elements of corneal infiltration and pannus in a varying degree. There are, therefore, three types of lesions: one, in which infiltration with consequent clouding and thickening of the cornea is especially marked, another in which the the vascular reaction and pannus are the most noticeable features, and a third which combines the two processes in about equal proportion.

Three early cases of the vascular and the infiltrative types of keratitis are illustrated in Figs. 9 to 12. The eye in Fig. 9 shows a well marked ciliary injection with a delicate network of vessels extending over the cornea. The area covered by this pannus was faintly clouded, but outside of this zone it was perfectly clear. Fig. 10 shows a condition of essentially the same character except that in this case the lesion arose from the inferior margin of the corner.

In contrast to these, the eye in Fig. 11 shows a crescent-shaped area of slight opacity with a small opaque dot at its center (descemetitis) which extends from the limbus over the margin of the pupil. At this time, the vessels were just beginning to appear at the corneal margin; 24 hours later (Fig. 12), the infiltration had increased very markedly, forming an opaque, elevated area with a narrow but dense pannus at its outer margin. The relative proportion between the vascular and infiltrative reactions shown in these three cases was maintained throughout their development.

Attention may be called to the ciliary injection and inflammatory reaction in the conjunctiva associated with these, which is well shown in Figs. 11 and 12.

When fully developed, the picture presented in cases of keratitis was very variable. The classical conditions are those presented in Figs. 13 and 14 which show pronounced keratitis involving a large part of the cornea. The first of these (Fig. 13) shows three things: first, a diffuse clouding of the cornea extending well below the pupillary margin; second, dust-like particles of dense opacity which are best seen over the pupil; and third, a very marked and uniform pannus, which extends like a curtain over the upper portion of the cornea.

The second lesion is quite different from this. As in the preceding case, there is a milky clouding of the cornea with granular deposits on the posterior limiting membrane but the noticeable feature of difference is the absence of a well defined pannus or even of a marked pericorneal injection. There were, however, a few vascular filaments in the cornea and a faint pannus can be seen towards the anterior or internal angle of the eye. As seen in these two animals, descemetitis was usually present in cases of marked corneal involvement but was frequently absent in milder ones.

Two older lesions of the cornea which will serve to complete the picture of this condition are reproduced in Figs. 15 and 16. The first of these affections (Fig. 15) was of 6 weeks duration and is given especially to illustrate the deep vascularization of the corneal lesion which frequently occurs during its later stages. The marked interstitial infiltration and descemetitis are quite obvious, and attention may be called also to the granulomatous lesion in the fornix.

The second lesion of this group (Fig. 16) had an unusual history of repeated relapses extending over a period of 27 months and is used to illustrate the formation of the salmon patch which is less constant in cases of keratitis in the rabbit than in man. The irregular area of dense opacity which extends from the upper margin of the cornea across the pupillary area developed 15 months after the first attack of keratitis. It was at first of a reddish gray color, subsequently changing to a pale orange, then yellow and gray. These appearances changed from time to time with the recurrent activity of the eye infection. Eventually the opacity diminished to a considerable extent, but a diffuse haziness of the cornea with a central opacity was still present when the animal was killed 27 months after the lesions first appeared.

Punctate areas of clouding or opacity, unaccompanied by a vascular reaction of any kind, have been observed in the central portion of the cornea in a few instances. We have not been able to investigate these lesions, however, and nothing is known as to their cause or nature.

Briefly, therefore, the only known form of syphilitic keratitis in the rabbit is an interstitial keratitis with pannus which usually arises from the superior margin of the cornea but may develop in other positions or as a circumcorneal affection. Ordinarily only a portion of the cornea is involved and the alterations produced are relatively slight, but in more pronounced cases, the entire cornea may be affected, with the production of deep seated lesions which may persist for months or even years.

The process of resolution of corneal affections was an irregular one. Usually the infiltration was first to disappear, leaving a vascular network as the only mark of the previous lesion. This is well brought out by a comparison of the eyes in Figs. 15 and 6, Fig. 6 representing a period 38 days later than Fig. 15. The aberrant vessels were at times very slow to disappear and have been known to persist almost unchanged for several months. In exceptional instances, the vessels disappeared before the infiltration, or resolution occurred in both directions at about the same rate. As a rule, resolution was complete, leaving no mark of the previous lesion.

### *Iritis.*

Apparently iritis has not been recognized as a manifestation of generalized syphilis in the rabbit distinct from that of the cornea, except in the one instance recorded by Reasoner of a gumma which involved the ciliary body, the iris, and the lens. Among the animals studied by us, however, it was a very common affection. Case records show a higher incidence of keratitis, but when the nature of the lesions and the relative ease or difficulty of their detection are considered, it appears quite likely that iritis was the more common of the two. The majority of the cases were recorded in albino rabbits, and while we were inclined to attribute this to difficulties of diagnosis interposed by a pigmented iris, it must be recognized that the nature

of the iris may be an important factor in determining the occurrence of these lesions.

The conditions which we have classed as iritis varied from an acute hyperemia of relatively short duration to plastic and granulomatous processes which produced permanent lesions of the iris. While these affections are spoken of as iritis, it must be understood that in many cases the ciliary body as well as the iris was involved, but the exact location of the lesion could not be determined clinically so that the term iritis is used to cover a group of reactions which was characterized chiefly by readily recognizable changes in the iris. Microscopic examination of a number of eyes showed, however, that the lesion was at times practically confined to the base of the ciliary body, while in other cases the ciliary body, the iris, and the choroid were all involved.

The most common form of iritis was an acute diffuse affection which lasted for only a few days. The usual signs in these cases were photophobia and profuse lacrimation with conjunctival and ciliary injection which appeared before any definite alteration could be detected in the iris and lasted until the acute reaction began to abate. The appearance presented in such cases may be seen by comparing Figs. 17 and 18 which show the affected and the normal eyes of an animal at the very beginning of an attack of iritis and Figs. 19 and 20 which show respectively a slightly later stage of iritis and a photograph of the same eye taken 1 week later. (Both eyes of this animal were involved at the same time.)

The changes in the iris itself began with an acute hyperemia. In albino rabbits, the marginal and axial vessels stood out prominently, while the substance of the iris appeared but little altered (Figs. 17 and 21). As the condition progressed, the vessels became less conspicuous while the color of the iris deepened to a dull red, a cyanotic, or rose color (Figs. 17, 19, 21 and 22); occasionally small hemorrhages occurred (Fig. 22) and the structural details of the iris became blurred and indistinct (*cf.* Fig. 18 with Figs. 17 and 19 to 22).

This condition lasted for a very short time, as a rule (24 to 72 hours), the vascular dilatation then subsided, and the diffuse discoloration gradually disappeared, leaving the iris slightly more clouded than normal (*cf.* Figs. 18 and 20).

Meantime, certain alterations in the pupillary reaction were noted. Under the conditions of lighting used, the normal pupil measured from 7 to 9 mm. in diameter; with the development of an acute iritis, the pupil gradually contracted until it measured only 4 to 6 mm. in diameter (*cf.* figures) and reacted very sluggishly to light (flash). The pupil and the pupillary reflexes usually returned to normal as the iritis subsided.

In darkly pigmented irides not all of these changes could be detected. The conditions usually noted were a change in the color of the iris, clouding or opacity, and a narrowing of the pupil. These may be made out by comparing the infected and the normal eyes of the same animal in Figs. 23 and 24 respectively. In this instance, it can be seen that the iris of the infected eye is distinctly mottled and of a lighter color, as well as hazy, and that the pupil is narrower than that of the normal eye; this was the condition usually found in this class of animals.<sup>1</sup>

In the group of cases described, the contents of the anterior chamber were little if at all affected and there was no descemetitis. In a few instances of severe iritis, there were hemorrhages into the anterior chamber (see Fig. 16), or the aqueous was slightly clouded (Fig. 22) and there were granular deposits on the posterior limiting membrane; these are just visible in Fig. 23.

There were also a few cases of plastic iritis. These differed from the condition which has been described in the presence of a slight exudate, which appeared to form on the posterior surface of the iris and protruded beyond the pupillary margin as a grayish white or yellowish white film. In some instances, the pupil was contracted, irregular, and fixed, while in others it retained its normal shape and its accommodation to light was less impaired.

<sup>1</sup> In comparing the pupils and pupillary reactions of rabbits, it should be noted that in the ordinary diffuse light of the laboratory, there is a decided difference in the size and the accommodation to light between an albino rabbit and one with a dark colored iris. The pupil of the albino is relatively smaller and reacts quickly to light (flash), contracting to a very small diameter. The pupil of an animal with a dark colored iris reacts more slowly, and the extent of the contraction is much less—sometimes barely perceptible.

The inflammation in this group of cases also cleared up within a short period of time and the exudate was absorbed without the formation of fibrous synechias.

The diffuse congestive affections of the iris appeared to be referable to a lesion at the base of the ciliary body rather than of the iris itself. It was only in the more pronounced cases and in instances in which hemorrhage or exudative phenomena were present that definite lesions of the iris could be detected, and even in these the ciliary body appeared to be more affected than the iris, but this could not be determined clinically.

Diffuse affections of the iris merged by insensible degrees with another type of affection in which the main lesions were of a focal nature. Fewer of these have been seen and comparatively little is known of them. The most common condition observed was a focal area of congestion or hemorrhage, an example of which is given in Fig. 25. These lesions were single or multiple but produced no alteration in the iris except at the points involved, and symptoms of acute iritis were usually absent.

A second condition, which was more characteristic, is that shown in Fig. 26. These cases presented all of the manifestations of an acute diffuse iritis, but in addition, small granulomatous nodules developed in the substance of the iris, usually near the pupillary border. There was partial or complete fixation of the iris with some irregularity of the pupil.

The pendant nodule seen in Fig. 26 developed in the superior margin of the pupil but in some way became torn loose except at one point, leaving a wedge-shaped defect in the iris which is plainly visible. A second nodule is seen above and to the right of the point of attachment of the first.

As a rule, the granulomatous lesions were comparatively small and tended to resolve without the production of any marked injury to the iris. In one instance, however, a lesion of this type developed into a large granulomatous mass which involved a considerable portion of the iris and filled nearly half of the anterior chamber of the eye. This lesion is shown in Fig. 27.

The formation of posterior synechias resulting in permanent alterations of the pupil was very rare. In one animal of our series, this



condition developed very early and there was marked irregularity and almost complete occlusion of both pupils which lasted up to the time of the death of the animal—a period of nearly 2 years. The right eye of this animal is shown in Fig. 28. The dark area at the center of the eye represents the outline of the original pupil but at the time this photograph was taken, most of this area had been filled in with granulation tissue and the only aperture present was of very small size and can just be seen at the lower and anterior edge of the corneal opacity.

### *Clinical History.*

*Correlation of Eye Affections.*—The clinical history of the eye affections described as pericorneal injections, conjunctivitis, keratitis, and iritis contains many points of interest. They are so intimately related to one another that they can hardly be considered as entirely distinct forms of infection. One condition may occur without the others but the circumstances were such as to favor a combination of the various affections. Thus, pericorneal injection and conjunctivitis may occur in the absence of any definite lesion of the cornea or iris, but the latter conditions were always preceded or accompanied by pericorneal injection and conjunctivitis. In like manner, iritis and keratitis may occur independently, or they may occur together, or one condition may follow the other. This peculiar association rests upon a simple anatomical basis. Histologically, it was found that these affections could all be traced to a common lesion which was centered about the vessels which encircle the cornea. This lesion first makes its appearance in the wedge-shaped mass of loose connective tissue at the outer side of the sclerocorneal junction (episcleral tissues). The vessels in this area become dilated, the tissue is edematous and infiltrated with polyblasts. If the lesion is confined to this area, it manifests itself in the form of a ciliary injection. The infection tends to spread, however, extending to the conjunctiva, to the substance of the cornea, or inward towards the canal of Schlemm and the spaces of Fontana. This led to the development of lesions in the cornea, the ciliary body, the iris, and occasionally in the choroid, giving rise to clinical signs of infection, the nature of which depended upon the parts thus involved.



When viewed from this standpoint, the eye affections described assume a less complex character.

*Predisposing Factors and the Relation to Other Manifestations of Infection.*—As was mentioned in connection with keratitis, affections of the eyes may be very frequent or very rare with a given strain of *Treponema pallidum*, depending upon a variety of conditions. From an analysis of the circumstances under which eye infections occurred in our series of animals, it was found that in more than 75 per cent of the cases, they were the only generalized lesions which occurred or were the last type of lesion to appear. In other words, eye lesions appeared to occupy a terminal position in the sequence of tissue reactions.

It was also found that experimental conditions could be employed which would emphasize this relation. These cannot be discussed further than to say that in general, circumstances which were unfavorable for the occurrence of other generalized lesions, tended to increase the relative incidence of affections of the eyes and conversely, those conditions which were most favorable for the occurrence of other lesions tended to reduce the relative incidence of eye lesions. Thus, double inoculation and late castration produced a high percentage of eye affections while unilateral inoculation and early castration reduced the incidence of these lesions.

*Occurrence and Duration.*—Considered collectively, eye lesions occurred at about the same interval of time after inoculation as other generalized lesions. The first lesions usually appeared at from 2 to 3 months after inoculation. Pericorneal injection and conjunctivitis were among the early affections, while keratitis and iritis occurred slightly later on the whole and the majority of the cases appeared near the end of the 3 months period. In several instances, eye lesions did not appear for as much as 6 to 8 months after inoculation, and in one animal, the interval was 2 years and 3 months, and in another, approximately 3 years.

One or both eyes might be involved, either simultaneously or in rapid succession. Less often, there was a considerable interval between the appearance of the two lesions. The duration of the affection was very short as a rule; simple pericorneal injections, diffuse conjunctivitis, and iritis frequently lasted for only a few days but

were of longer duration when complicated by lesions of the cornea. Keratitis was the most enduring, but even this rarely lasted longer than 2 to 3 weeks. The most marked corneal lesions lasted for several months, and in the one instance described above the lesions had not cleared up when the animal was killed 33 months after inoculation.

*Recurrence.*—Exacerbation of partially resolved lesions of the eyes or recurrence of completely healed lesions was almost the rule among those animals which were held over long periods of time. Two or three attacks of iritis and especially of keratitis were quite common. In one animal, numerous attacks of keratitis and iritis occurred over a period of approximately 2 years. Actual count of the attacks was lost but the number may be safely placed at more than a dozen. Several additional instances were recorded in which recurrent lesions appeared more than a year after inoculation.

Relapse of partially or completely healed lesions of the eyes occurred more often than in any other class of affections. The tendency to relapse in the case of keratitis has been noted by numerous observers and is the one redux phenomenon of the experimental infection which has received general recognition.

The tendency of eye lesions to relapse as well as the peculiar circumstances of their occurrence is probably to be explained by the degree of protection afforded these parts by reactions elsewhere and the feeble protection afforded by the reaction developed in these tissues.

#### SUMMARY AND CONCLUSIONS.

From the study of a number of instances of eye infection in the rabbit, it was found that a variety of affections might occur following scrotal or testicular inoculations of *Treponema pallidum*. Those observed included ciliary injection, conjunctivitis, keratitis, and iritis which might occur separately or in combination with one another, except that keratitis and iritis were always accompanied by a reaction in the ciliary vessels and usually by a conjunctivitis.

Several forms of each of these affections were described, and while some of them were regarded as presenting a very characteristic picture, it was recognized that the conditions present in other cases

were not sufficiently distinctive to permit of a clinical diagnosis. With a few exceptions, however, the pathology of the lesions was sufficient to identify them as processes of a syphilitic nature.

It was also found that this group of lesions usually arose from a common focus of infection which was located in the episcleral tissues immediately surrounding the cornea. From this point, the infection tended to spread to the conjunctiva and the cornea, or toward the canal of Schlemm and the spaces of Fontana and thence to the ciliary body, the iris, and the choroid. The localization of the lesion and the mode of extension were held to be responsible for the combination of manifestations usually observed.

From an analysis of the circumstances under which affections of the eyes occurred, it was found that the great majority of them occupied a definite position in the scheme of tissue reactions, being the only generalized lesions developed or the last type of lesion to appear.

These facts, together with the unusual frequency of relapse in these affections, were believed to indicate that a low degree of protection was conferred upon these tissues by reactions taking place elsewhere and that the protection afforded by the local reaction was of a feeble character. This deduction was in part confirmed by the fact that it was found to be possible to increase or decrease the incidence of eye lesions by the use of experimental means which varied the scheme of reaction in animals inoculated with a given strain of *Treponema pallidum*.

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## EXPLANATION OF PLATES.

The illustrations are reproductions of photographs which represent the objects at their natural size. Unless otherwise indicated, statements of time are estimated from the date of inoculation.

## PLATE 7.

FIGS. 1 to 8. Ciliary injection, and affections of the conjunctiva and episcleral tissues.

FIG. 1. 61 days. A typical example of ciliary injection with acute conjunctivitis.

FIG. 2. 88 days. Circumcorneal injection (ciliary) with acute conjunctivitis.

FIG. 3. 132 days. Acute diffuse conjunctivitis with pronounced swelling and intense redness of the conjunctiva. There is also a moderate pericorneal injection.

FIG. 4. 54 days. Miliary granulomata of the pericorneal tissues associated with ciliary injection.

FIG. 5. 64 days. A phlyctenular lesion of the conjunctiva and episcleral tissues.

FIG. 6. 156 days. A syphilitic granuloma involving the conjunctiva and episclera. There is also an old lesion of the cornea which is undergoing resolution.

FIG. 7. 45 days. A pericorneal nodule of a type which may or may not be syphilitic. Note the absence of a vascular reaction.

FIG. 8. 49 days. Multiple lymphoid nodules in the pericorneal tissues which apparently are not of syphilitic origin.

## PLATE 8.

FIGS. 9 to 16. Lesions of the cornea.

FIG. 9. 54 days. Interstitial keratitis. Ciliary injection and early vascularization of the cornea.

FIG. 10. 100 days. An early keratitis of the lower margin of the cornea analogous to that in Fig. 9.

FIG. 11. 95 days. An early keratitis of the infiltrative type. There are a well marked pericorneal injection and conjunctivitis with a slight descemetitis.

FIG. 12. 24 hours later. There are an increase in the inflammatory reaction and a well developed pannus at the margin of the cornea.

FIG. 13. 73 days. A typical example of interstitial keratitis with marked pannus.

FIG. 14. 106 days. An example of interstitial keratitis showing marked infiltration of the cornea with comparatively slight vascularization.

FIG. 15. 133 days. Interstitial keratitis involving the entire cornea with deep vascularization.

FIG. 16. 1 year, 8½ months. Keratitis with well marked salmon patch covering large area of cornea. The dark spot in the eye is due to hemorrhage in the iris.

## PLATE 9.

FIGS. 17 to 28. Affections of the iris.

FIG. 17. 94 days. Acute iritis showing drooping of the upper lid, slight lacrimation, and slight contraction of the pupil.

FIG. 18. 94 days. Normal eye of the same animal as that in Fig. 17 given for comparison.

FIG. 19. 70 days. Acute iritis, partial closure of the lids, and lacrimation. Narrowing of the pupil and clouding of the iris.

FIG. 20. 1 week later. Same eye as that in Fig. 19. Photograph shows partial recovery from the iritis. Eye appears normal except for the loss of structure in the iris. Cf. with Fig. 18.

FIG. 21. 132 days. An early acute diffuse iritis. There is a well marked pericorneal injection. Vessels of the iris are engorged and its substance is clouded. There is also clouding of the contents of the anterior chamber. The right eye of this animal is shown in Fig. 3.

FIG. 22. 125 days. Acute diffuse iritis, or slightly later stage than that shown in Fig. 21. Vessels are no longer visible but the iris is distinctly clouded and there is a focus of hemorrhage immediately above the pupil.

FIG. 23. 85 days. Acute iritis in an animal with pigmented iris. Cf. with Fig. 24. The iris is of a lighter color than normal, somewhat mottled, and the structure indistinct. Pupil is also contracted.

FIG. 24. 85 days. Normal eye of the same animal as that in Fig. 23.

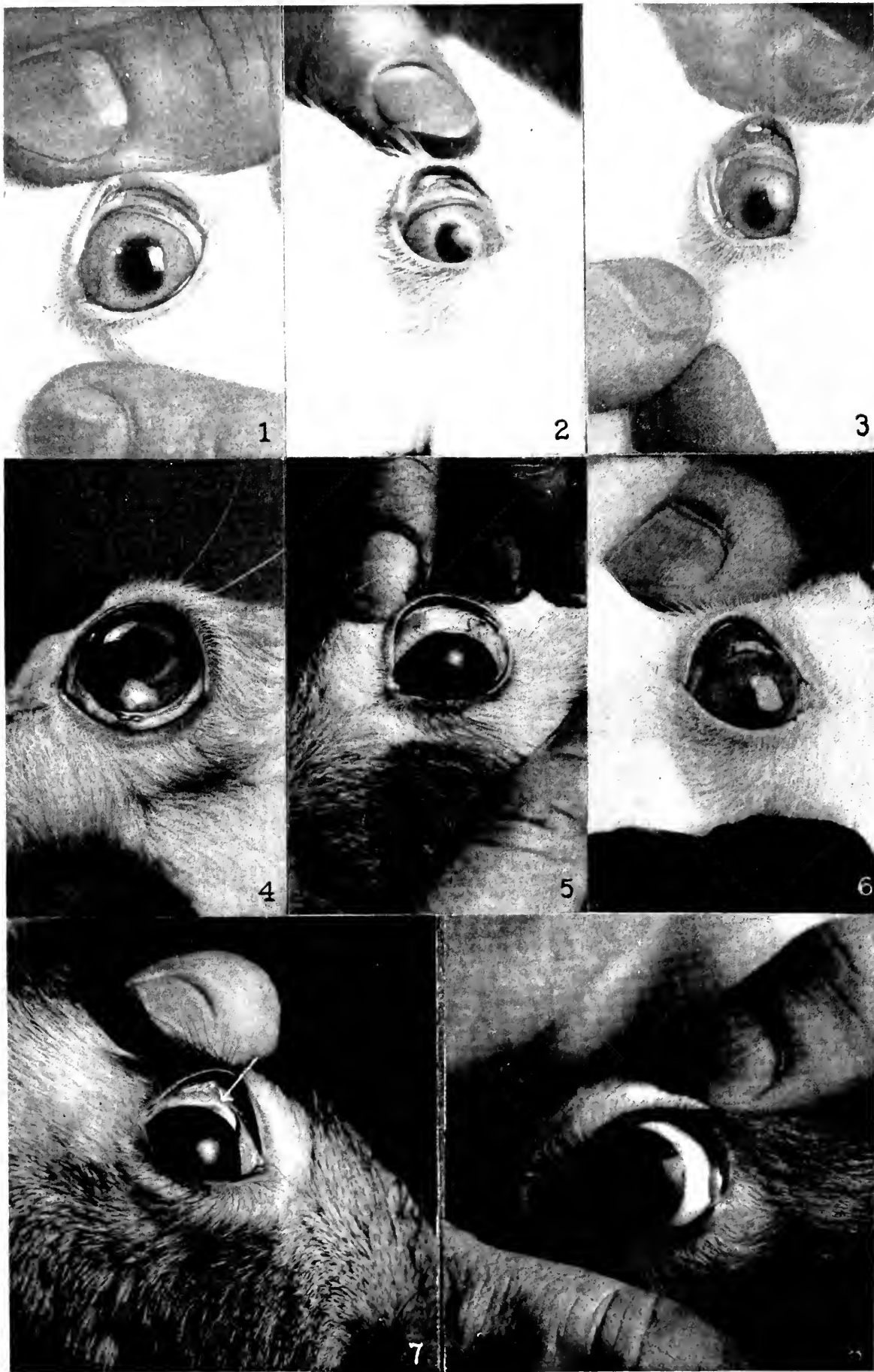
FIG. 25. 95 days. Focal lesion of the iris. Remainder of the iris appears entirely normal.

FIG. 26. 82 days. Acute diffuse iritis with a granuloma, superior margin of the pupil. The small nodule seen at the upper margin of the iris has been torn loose from its original position, leaving defect in the margin of the pupil.

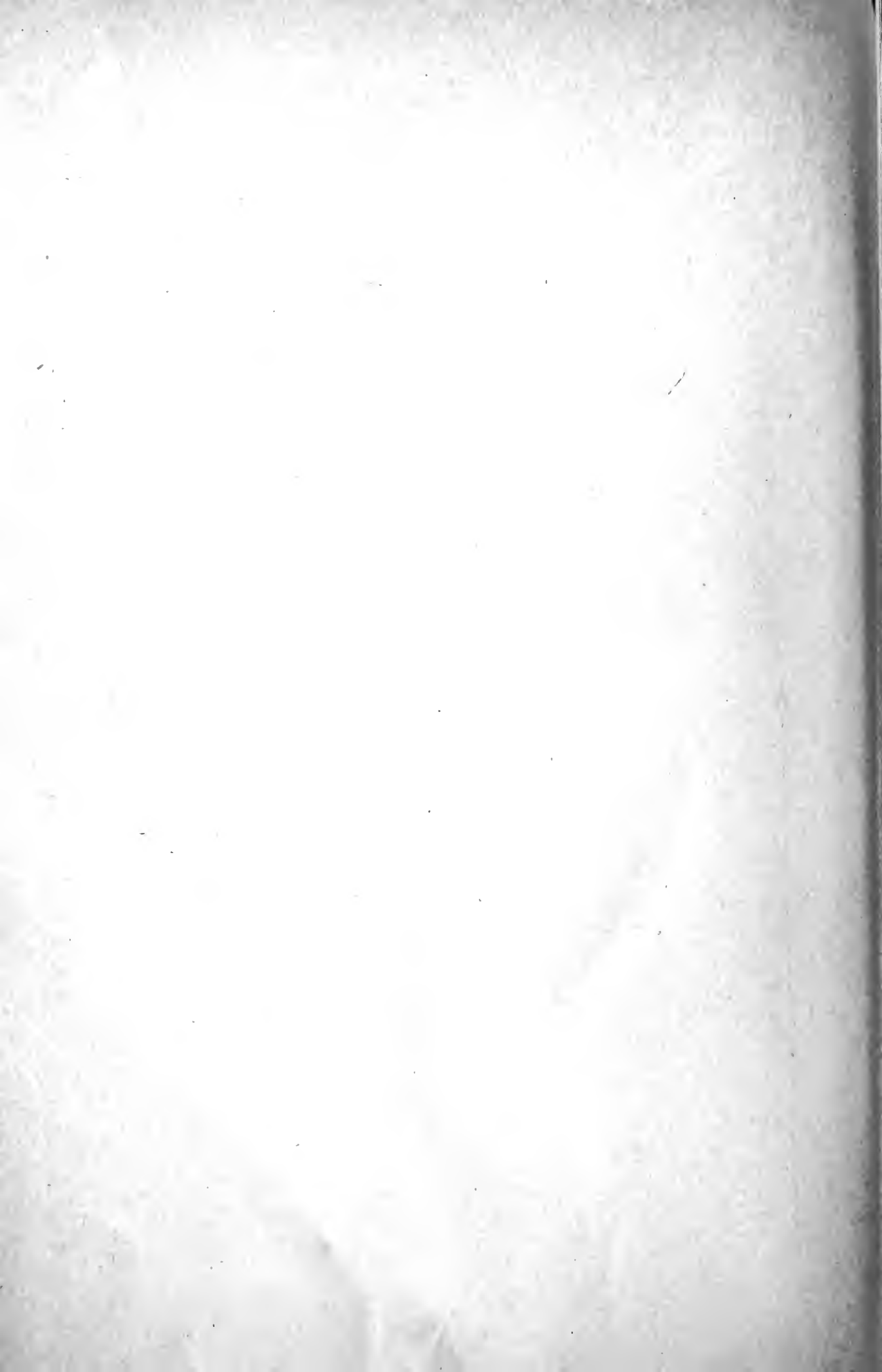
FIG. 27. 185 days. Large granulomatous lesion of the iris projecting into the anterior chamber of the eye.

FIG. 28. 1 year, 7 months. Occlusion of the pupil with irregularity and fixation. Permanent lesion.

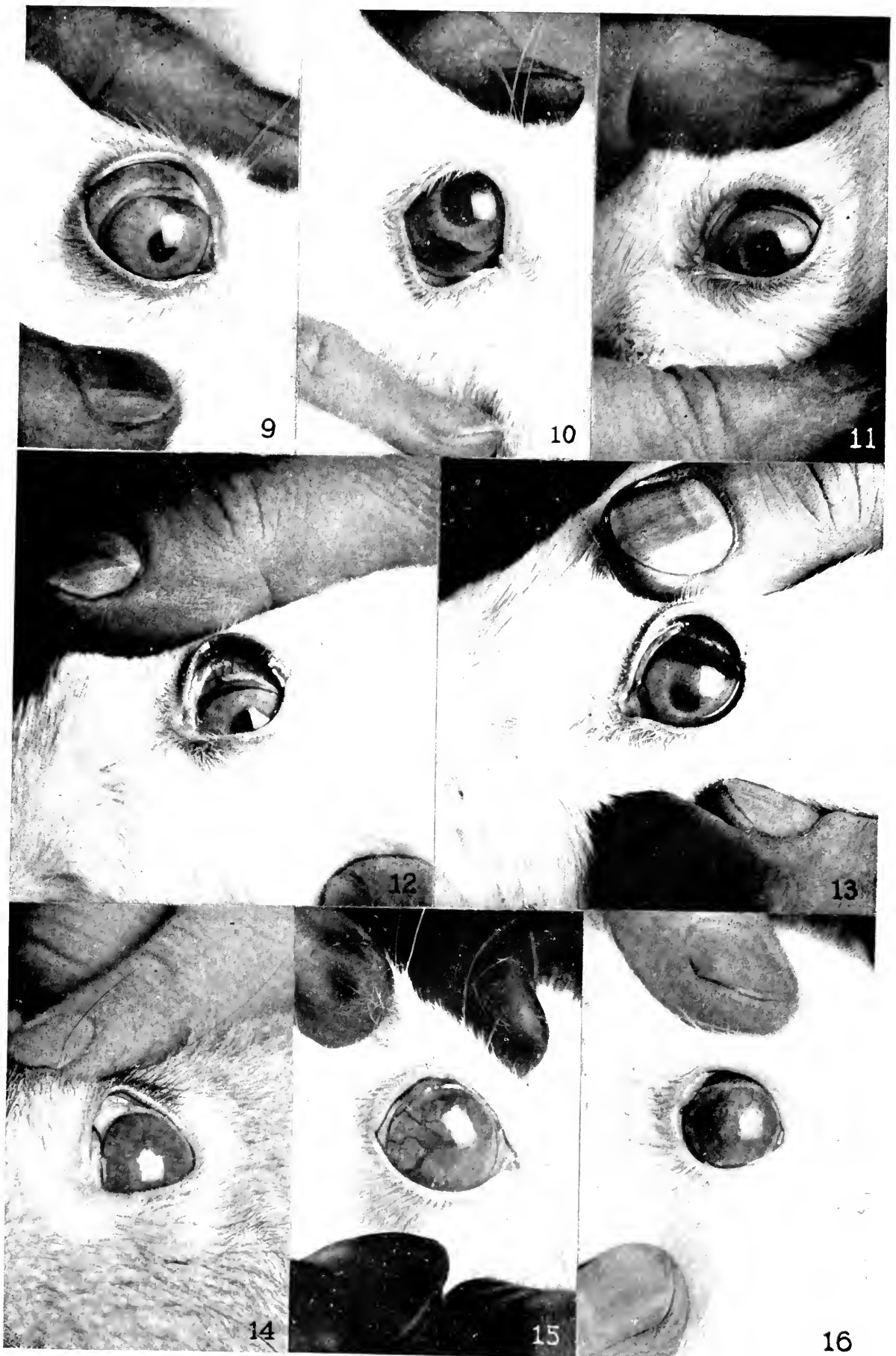
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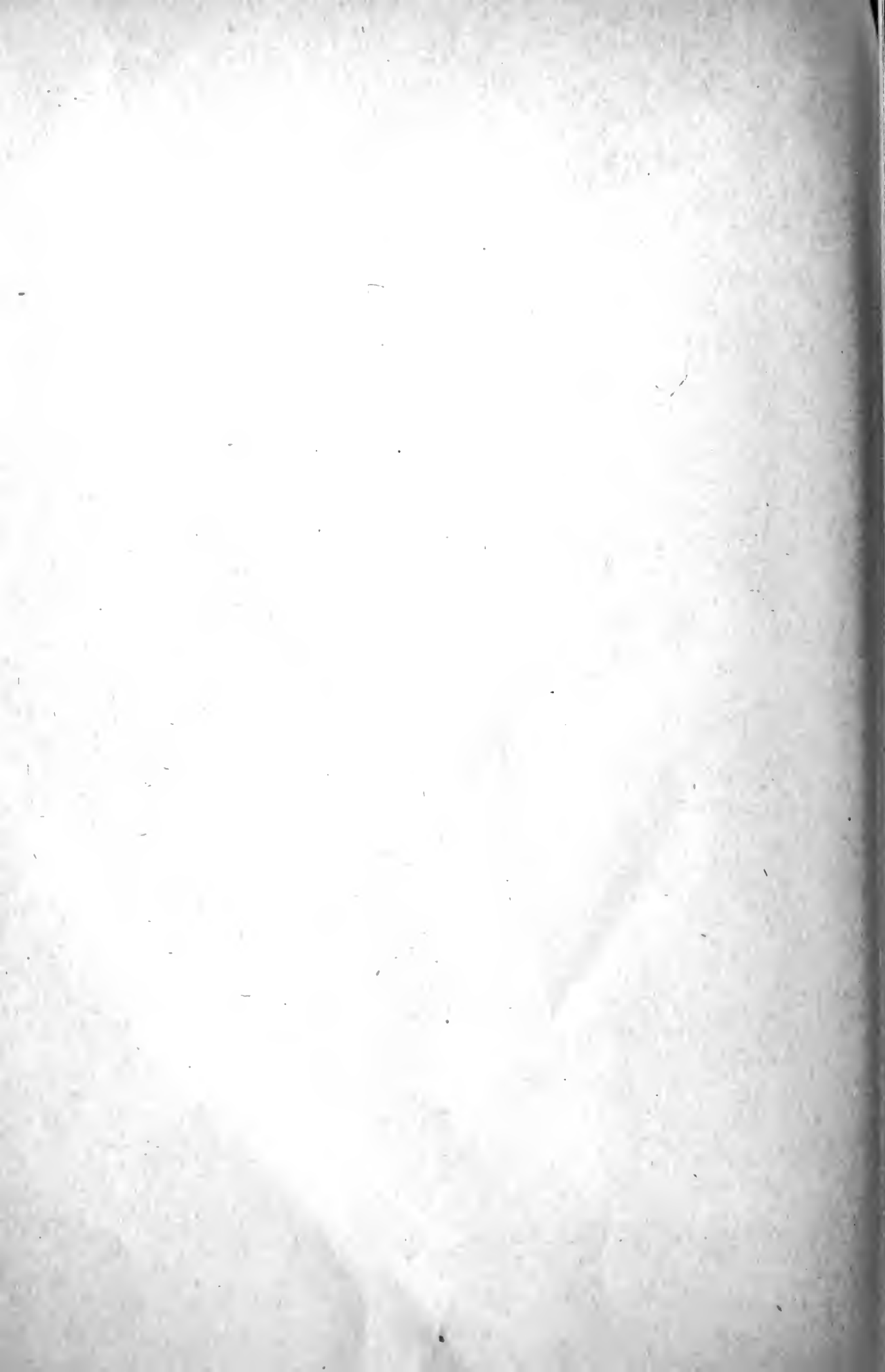
(Brown and Pearce: Experimental syphilis in the rabbit. VII.

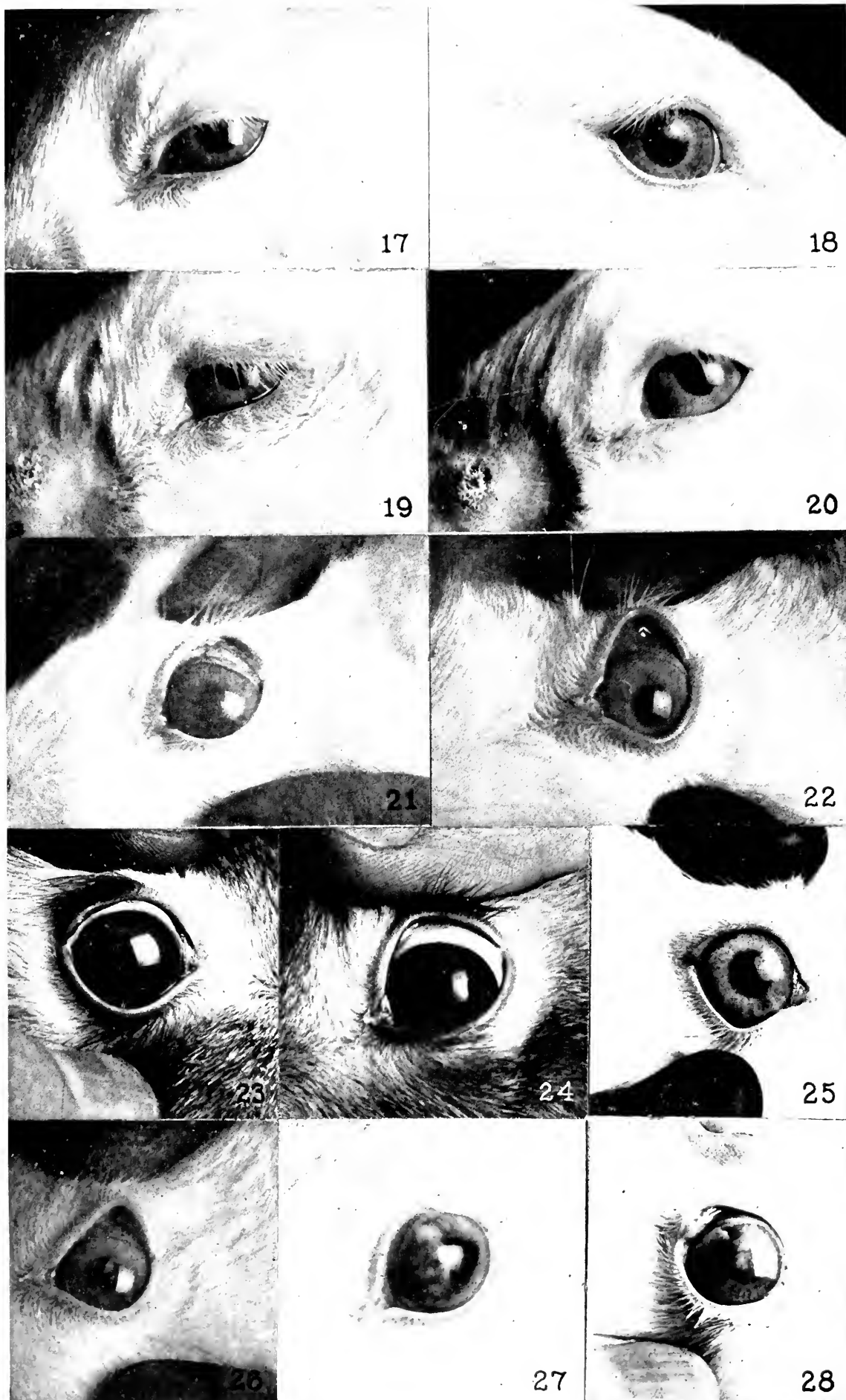






(Brown and Pearce: Experimental syphilis in the rabbit. VII.)







NOTE ON THE PRESERVATION OF STOCK STRAINS OF  
TREPONEMA PALLIDUM AND ON THE DEMON-  
STRATION OF INFECTION IN RABBITS.

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The maintenance of stock strains of *Treponema pallidum* for teaching or experimental purposes has been both time-consuming and expensive, due to the necessity for constant watchfulness and frequent transfer from animal to animal in order to guard against loss of strains, but no way has been found to overcome these difficulties, and in spite of the greatest care, valuable strains are frequently reported as lost.

In like manner, the usefulness of *pallidum* infections in rabbits for experimental purposes has been subject to serious limitations on account of the lack of reliable means for determining end-results as regards infection when no demonstrable lesions were present. This has been especially true in chemotherapeutic investigations in which the method for determining a cure, and the only one available, was more or less prolonged clinical observation. It has been assumed that healing of existing lesions without recurrence within a short period of time (1 to 3 months) constituted evidence of cure and the results of all experiments thus far reported have been based upon the use of criteria of this kind.

The supposed necessity for frequent transfers, the presumed loss of stock strains of *Treponema pallidum*, and the supposition that freedom from lesions constituted evidence of cure were all based upon the belief that *pallidum* infections in the rabbit are self-limiting—that with the healing of the lesions the infection also became extinct. Numerous isolated observations by ourselves and others have cast considerable doubt upon the validity of these earlier views, but until quite recently, no systematic experiments were carried out for the

purpose of determining the exact nature of the infection in the rabbit as regards dissemination of organisms, the duration of the infection, and the possibilities of recovering the virus from animals after all manifestations of disease had disappeared.

During the past 2 years, a large series of experiments has been carried out in an attempt to obtain definite information upon these points. One of the methods employed was that of test inoculations of normal animals with material from superficial lymph nodes of infected animals. Parts of this work have been reported in connection with studies on the dissemination of spirochetes (1, 2) and the demonstration of spirochetes in the lymph nodes during latent periods of infection (3).

The details of these experiments need not be repeated; it will suffice to state that test inoculations have been made from inguinal or popliteal nodes of 51 rabbits with positive results in all instances. The material studied included four classes of animals: (1) animals with developing or active infections of from 48 hours to 2½ years duration; (2) animals with latent infection in which no lesions had been present for from 3 months to 2 years with a period of infection ranging from 7 months to 4 years and 3 months; (3) drug-treated animals in which no lesions had recurred during a period of 3 months observation; (4) animals used for serial passage of *Treponema pallidum* from lymph node to testicle over a period of about 14 months. The majority of the tests were carried out during the first 3 months of the infection.

Before it could be concluded that the organisms recovered were localized in the tissues, it was necessary to exclude the blood as a possible source. During early stages of the infection, this could be done only upon the basis of relative infectivity. Later, however, blood inoculations were uniformly negative while the lymph nodes gave positive results.

The experiments cited showed that with old strains of *Treponema pallidum*, generalization and localization of the organisms in lymphoid tissues are a constant phenomenon of the infection; they also indicated that the infection is permanent and that the treponema can be recovered at any time by inoculation of material from superficial lymph nodes of infected animals. It thus appears that while rabbits acquire a high degree of protection against the toxic effects of *Treponema*

*pallidum*, they are no more capable of terminating the infection than is man.

Whether the same conditions hold true for recently isolated strains is not entirely certain, but since the latter exhibit the same tendencies to lymphoid involvement as the older strains, it is not unlikely that they too are capable of surviving in the rabbit for an indefinite period of time.

Once it has been shown that there is a constant and permanent localization of *Treponema pallidum* in the lymphoid tissues of infected animals, there are many applications of these facts which are quite obvious, and the possibility of utilizing them in connection with the preservation of stock strains and as a means of determining the presence of infection in experimental animals appeared to be of sufficient importance to warrant a special note upon this subject.

The method proposed for the preservation of stock strains of *Treponema pallidum*, when not in active use, is merely to keep a sufficient number of infected animals to guard against loss of the strain by their death. Serial transfers may be dispensed with. When it is desired to recover the organism for teaching or experimental purposes, a popliteal node may be excised with aseptic precautions, minced, and ground in a mortar; an emulsion is then prepared by the addition of about 1.5 cc. of sterile normal salt solution with further grinding. The resulting fluid is aspirated into a syringe fitted with a No. 22 gauge needle and about 0.5 cc. of the emulsion is injected into a testicle of one or more normal rabbits. In order to allow ample time for the development of a testicular infection, the inoculation should be made 6 to 8 weeks before the organism is needed.

Exactly the same method is applicable to the demonstration of infection in experimental animals. The circumstances should determine in each case when test inoculations are to be undertaken. In chemotherapeutic experiments, for example, it would appear to be advisable to follow the old system of clinical observation for at least 1 to 2 months before resorting to test inoculation; otherwise an infection which had been almost extinguished might not be given a sufficient opportunity to reestablish itself.

The essential requirements of the method are extremely simple: One is advised against complicating the technique by the use of



foreign substances to aid in the grinding of material; filtration is unnecessary; the injection of large amounts of fluid tends to produce inflammatory reactions in the testicles which may obscure subsequent lesions, and the use of strong antiseptics, either in the removal of lymph nodes or in making inoculations, is contraindicated.

Eberson and Engman (4) have used a similar method with success in demonstrating infection in the lymph nodes of human subjects with latent syphilis, thus establishing another analogy between the human and animal infection.

Positive results from testicular inoculation are usually not difficult to determine. It should be noted, however, that atrophy of the testicle may occur instead of the usual granulomatous enlargement and that in exceptional instances, infection may be recognized by the development of an adenopathy when no lesions can be detected at the site of inoculation.

By the use of the method described, a great saving in time and expense may be accomplished, and infection can be determined with comparative ease and with much greater certainty than was hitherto possible. However, until the delicacy of the method has been subjected to further test, negative results are still to be accepted with reserve.

#### SUMMARY.

Experiments carried out on rabbits infected with *Treponema pallidum* showed that there was a constant invasion and localization of the organisms in the superficial lymph nodes, that the infection persisted indefinitely, and that organisms could be recovered at any time from such nodes as the popliteals. Based upon these observations, a method is proposed for the preservation or recovery of stock strains of *Treponema pallidum* and for the demonstration of infection in rabbits.

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## EXPERIMENTAL RICKETS IN RATS.\*

### I. A DIET PRODUCING RICKETS IN WHITE RATS, AND ITS PREVENTION BY THE ADDITION OF AN INORGANIC SALT.

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PLATES 10 TO 17.

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It is remarkable that, in the experimental study of rickets from the dietary point of view, the rat has been so little used as an experimental animal. With the exception of the recent papers of McCollum and his associates, we find no record of a direct attempt to induce rickets in these animals by modification of the diet.

This is the more surprising in view of the fact that the occurrence of rickets in rats maintained under laboratory conditions has been known to pathologists since the publication of Morpurgo (1) in 1901. The essential identity of the lesions with those of human rickets has been amply demonstrated by Morpurgo (2) himself, by Schmorl (3), Weichselbaum (4), Hohlbaum (5), Iselin (6), Pappenheimer (7), and especially by the detailed studies of Erdheim (8). None of these workers standardized or controlled the diet of the animals which they studied. Morpurgo was interested in the supposed infectious origin of the disease; Iselin, Hohlbaum, and Erdheim, in its relation to the parathyroid gland; Pappenheimer, in the supposed influence of thymus extirpation on the production of rickets.

The recent paper of McCollum, Simmonds, Parsons, Shipley, and Park (9), which appeared while our work was in progress, deals specifically with the influence of deficient diets upon the production of rickets in rats. A number of diets are cited, deficient in various respects, upon which presumably the rats developed rachitic lesions. However, although the particular deficiencies are pointed out, it is not definitely stated which diets led to the development of rickets. Nor is reference made in the legends to the illustrations of rachitic

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\*Presented in abstract at the Meeting of the Society for Experimental Biology and Medicine, March 16, 1921.

lesions, to the particular diets which produced them. Since this paper is a preliminary one, one may assume that these data will be forthcoming in subsequent publications. The authors do not commit themselves to the particular dietary deficiencies concerned, stating<sup>1</sup> that:

“Any suggestion regarding the absence of a specific antirachitic substance or deficiency of either fat-soluble A or calcium as the primary agent in the production of rickets would be ill considered and might be far from the truth.”

In the second paper, by Shipley, Park, McCollum, Simmonds, and Parsons (10), the curative effect of the addition of cod liver oil to the diet is demonstrated. The two rations used in these curative experiments were No. 2638, “low in Ca, Na, and Cl ions, as well as in fat-soluble A;” and Ration 2677, containing an adequate amount of calcium, but “low in the fat-soluble A.” In this paper, a more definite statement is made as to the factors concerned in the production of the rachitic lesions, as follows:<sup>2</sup>

“Previous experience with the rat had taught us that by the use of faulty diets, especially certain diets deficient in the so called fat-soluble A or in both that substance and calcium, the cartilage and adjacent portions of the metaphysis of the long bones of the extremities could be rendered entirely free from calcium deposits and a condition identical with the rickets of human beings be obtained.”

There are obvious advantages in the use of rats for the experimental study of this disease. Aside from the close resemblance of the lesions to those of human rickets, the ease with which controls of the same litters can be obtained, the rapid development of the lesions, the fact that variations in susceptibility, possibly due to differences in breed, can be eliminated, the possibility of working with large numbers of animals, and the economy of space and expense, and finally the ease with which histological examinations of the bones can be carried out—all these considerations make the rat an ideal experimental animal for the study of the disease.

In continuing, by means of feeding experiments upon rats, the study of the mineral elements, which has engaged the attention of one of us for the past 15 years, we have found a relatively simple diet which, as far as our experience goes, leads regularly to the production of marked rickets. It has been further found that the introduction of 0.4 per cent secondary potassium phosphate ( $K_2HPO_4$ ) in place of an equal weight, *i.e.* replacing about one-seventh, of the

<sup>1</sup> McCollum, Simmonds, Parsons, Shipley, and Park (9), p. 340.

<sup>2</sup> Shipley, Park, McCollum, Simmonds, and Parsons (10), p. 344.

calcium lactate contained in the rickets-producing diet, has completely prevented the development of the rachitic lesions, although without influence upon the growth and body weight. Our experiences are summarized in Table I.

TABLE I.

Diet No.	Composition of diet.		Total No. of rats examined.	
			(a) Rachitic.	(b) Non-rachitic.
83	Patent flour,	95.0 per cent.	2	0
	Calcium lactate,	3.0 " "		
	Sodium chloride,	2.0 " "		
84	Patent flour,	95.0 " "	13	0
	Calcium lactate,	2.9 " "		
	Sodium chloride,	2.0 " "		
	Ferric citrate,	0.1 " "		
85	Patent flour,	95.0 " "	0	15*
	Calcium lactate,	2.5 " "		
	Sodium chloride,	2.0 " "		
	<i>Potassium phosphate</i>	0.4 " "		
	Ferric citrate,	0.1 " "		

\* Five of these placed on special diet at age of 60 days; one at 81 days; all remaining animals started at 4 weeks of age.

The diagnosis in each case has been based not only upon the gross changes found in the thorax and long bones at autopsy, but also upon the microscopic examination of several ribs (Figs. 1 to 5), incompletely decalcified (5 to 7 days in Müller's fluid). The histological criteria of rickets were: (1) the great increase in the width of the zone of preparatory cartilage, and its irregular projection towards the diaphysis; (2) the failure of calcium deposition in this zone of preparatory calcification; (3) the pronounced increase in the osteoid tissue, both in the region of the metaphysis and along the shafts of the bones. Most of the rats showed spontaneous infractions, visible especially on the internal surface as callus beads and especially numerous in the lower ribs near the chondrocostal junctions.

It was found that the diagnosis could be readily established during life by x-rays (Figs. 6 to 8). In the rachitic rats there was plainly seen in the head of the tibia a clear zone immediately beneath the

epiphysis, 1 or 2 mm. in width and concave towards the shaft. The normal controls or rats in which the potassium phosphate was included in the diet showed a distinct sharp line in place of this rarefied area.

### *Illustrative Protocols.*

*Rat 1750.*—Put on Diet 83 at 29 days of age.

#### *Record of Body Weight.*

Age, days.....	29	36	43	50	57	64	71	78	85	92	94
Weight, gm.....	59	46	49	50	54	49	52	52	49	52	Died.

*Gross lesions.*—Extreme pallor of eyes and mucous membranes. Slight corneal opacity. A little bloody exudate at inner canthi. *Skeletal system.*—Thorax: On the left side there is an extraordinary deformity. The costal margin is at a higher level than on the right side, and the liver shows a corresponding upward displacement. This is caused by a deep linear infolding of the ribs on the left side, beginning at the seventh and extending to the eleventh. The angulation is at the junction of bone and cartilage, and slightly external to the attachment to the left leaf of the diaphragm. There is no marked swelling of the chondrocostal junctions. Visible on the internal surface of the right wall of the thorax, about 2 to 4 mm. from the junctions of the bone and cartilage, there are pearly bead-like thickenings of the shafts of the ribs, extending from the fifth to the tenth rib inclusive. On the left side, the angulation, seen from within, appears to be just proximal to the cartilage, and there is the same nodular formation, which is more evident from the external surface, but is visible also from within. Long bones: Normal. *Lungs and abdominal viscera.*—Show no noteworthy change. *Spleen.*—Small and flat.

*Microscopic Examination.*—*Rib.*—There is great increase in the width of the zone of preparatory cartilage, which extends towards the shaft in the form of two deep prolongations in which the linear arrangement of the hypertrophic cartilage cells is entirely lost. Calcium deposition in the matrix of the cartilage is imperfect in the proximal portion and completely absent in the distal part. There is a dense spongiosa occupying the region of the metaphysis, and composed wholly of broad trabeculae of osteoid, with only an occasional central remnant of calcified bone. The blood vessels penetrate the cartilage irregularly. The cortex is broad, and the calcified bone surrounded both on its periosteal and endosteal surfaces by a wide osteoid margin. The bone marrow shows no fibrosis. *Femur.*—There is increased width of the proliferating cartilage at both extremities, with deficient calcification of the matrix. The spongiosa is dense, and the trabeculae are surrounded by wide osteoid zones. An excess of osteoid is present along the corticalis, especially about the perforating canals. Viscera show no lesions of interest.

*Diagnosis.*—Rachitis.

*Rat 2605.*—Put on Diet 84 at 28 days of age.

*Record of Body Weight.*

Age, days.....	28	31	38	45	52	59	60
Weight, gm.....	34	31	32	34	36	34	Died (weight 33).

*Gross Lesions.*—*Eyes.*—Cornea steamy at margin. Ingrowth of blood vessels from periphery of left eye. *Thorax.*—No gross deformity. There are definite early rachitic changes at the chondrocostal junctions; namely, increased width of zone of growing cartilage, perforating blood vessels, and irregular calcification in preparatory zone. *Femur.*—Cuts easily. Cartilage at lower epiphysis broader than normal. Spongiosa thickened. *Teeth.*—Opaque; hemorrhages in gums at roots of incisors. *Viscera.*—Normal.

*Microscopic Examination.*—*Ribs.*—Zone of preparatory calcification four to twenty-five cells deep with lateral projections into spongiosa. Latter composed exclusively of dense trabeculae of osteoid, surrounded by well formed osteoblasts; hyperemic marrow vessels. There is great excess of osteoid along the cortex.

*Diagnosis.*—Rachitis, marked.

*Rat 2603.*—Of same litter; maintained on Diet 84; also showed marked rachitic lesions.

*Rat 2611.*—Put on Diet 84 at 29 days of age.

*Record of Body Weight.*

Age, days.....	29	30	37	44	51	58
Weight, gm.....	35	33	33	35	37	Died (weight 34).

*Gross Lesions.*—*Eyes.*—Normal. *Thorax.*—No gross deformity or callus along ribs. Chondrocostal junction slightly beaded and definitely irregular, with increased translucency, and widening of zone of preparatory cartilage. Marked beading at vertebral extremity of ribs. Marrow hyperemic. *Femur.*—Shows broad zone of cartilage and cuts with less than normal resistance. *Teeth.*—Opaque. No deformity. *Viscera.*—Postmortem changes; not taken for section.

*Microscopic Examination.*—*Rib.*—(Müller's fluid for 5 days.) Zone of preparatory cartilage fifteen to twenty-five cells deep, irregular; calcification of matrix imperfect. Dense spongiosa near cartilage, composed almost wholly of osteoid surrounded by active osteoblasts; wide osteoid margin in shaft. Marrow spaces narrow; vessels hyperemic. Periosteum and perichondrium thickened over chondrocostal swelling.

*Diagnosis.*—Rachitis.

*Rats 2615 and 2617.*—Of same litter; maintained on Diet 84; also showed marked rachitic lesions.

*Rat 2606.*—Put on Diet 85 at 28 days of age.

*Record of Body Weight.*

Age, days.....	28	31	38	45	52	59	60
Weight, gm.....	34	33	36	36	38	35	Died (weight 33).

*Gross Lesions.*—*Eyes.*—Clear corneæ. *Thorax.*—No deformity, beading, or callus. Junction sharp and clear. Opaque zone of preparatory calcification visible. *Femur.*—Cuts with greater resistance than that of No. 2605. *Viscera.*—Normal. *Teeth.*—Opaque.

*Microscopic Examination.*—*Rib.*—(Müller's fluid for 5 days.) Zone of preparatory calcification four to eight cells deep. Calcium deposit in matrix normal. Slight irregular projections in one or two places. Ossification about cartilage fairly active; cortex thin, completely calcified—osteoid margin, not wider than normal controls of same age. Spongiosa rarefied, marrow very hyperemic.

*Diagnosis.*—Normal bones, showing inactive osteogenesis.

Rat is from same litter as Nos. 2603 and 2605.

Rat 2612.—Put on Diet 85 at 29 days of age.

#### *Record of Body Weight.*

Age, days.....	29	30	37	44	51	58	60
Weight, gm.....	39	37	39	42	42	43	Died (weight 38*).

\* May have lost weight after death.

*Gross Lesions.*—*Eyes.*—Normal. *Thorax.* No swelling of chondrocostal junction; line straight, with opaque transverse band corresponding to zone of provisional calcification. *Femur.*—Firm; normal epiphyseal line. *Viscera.*—Normal, save for congestion and patches of collapse in lungs.

*Microscopic Examination.*—*Rib.*—(Müller's fluid.) Practically normal line. Preparatory zone two to three cells deep. Adequate calcification of ground substance. Osteoid invisible; osteogenesis inactive. Marrow cavity wide and hyperemic. Spongiosa scant.

*Diagnosis.*—Normal bone.

Rat 2616, of same litter, maintained on Diet 85, showed normal bones, whereas Rats 2611, 2615, and 2617, the remaining members of the litter, on Diet 84, developed rickets.

#### *Calcium Content of the Bodies of Rachitic and Non-Rachitic Rats.*

Quantitative determinations of the total calcium contained in the bodies of rats of similar origin, feeding, and age to those described in the protocols, have also been made. Since not less than 99 per cent of the calcium in the body belongs to the bones, the calcium content of the body necessarily runs closely parallel with the growth and ossification of the skeletal tissue. A normal rat of 28 to 30 days of age may be expected to contain about 0.7 to 0.8 per cent of calcium, or about 0.3 gm. in a rat weighing about 40 gm. In rats which have been kept upon the experimental diets here described, the percen-

tages of calcium are somewhat more variable and their interpretation is complicated by the unavoidable differences in size of the animals, and, therefore, presumably in their calcium content at the time of placing upon the experimental diet, and by the fact that the final body weight is sometimes lower than the maximum weight attained by such an experimental animal. Nevertheless, in all comparable cases thus far examined, it has been found that Diet 85, although containing less calcium than Diet 84 has induced a larger assimilation and retention of calcium by the body, evidently as a result of the favorable influence of the potassium phosphate fed. This may be illustrated by the data of Rats 2613 and 2614 which were of the same litter as Rats 2611 and 2612 described in the protocols. Rat 2613, which had remained practically stationary in body weight (32 to 36 gm.) on Diet 84, contained 0.311 gm. of calcium, while Rat 2614, which also remained practically stationary in weight (34 to 38 gm.), but was fed Diet 85, showed 0.475 gm. of calcium. Thus, although growth in body weight was not induced by Diet 85, it did improve the assimilation of calcium to such an extent that the calcium of the body became fully 50 per cent higher than in the parallel case on Diet 84.

#### DISCUSSION.

It would be unprofitable to discuss at this time the specific factors in Diets 83 and 84, which may be responsible for the production of the rachitic lesions. These diets are inadequate for growth in the amount and character of the protein, as well as in their content of fat-soluble A. They contain liberal amounts of calcium lactate and sodium chloride and only a small amount of potassium. Experiments to determine the effect of adding various deficient substances to this basic rachitis-producing diet are now in progress.

The definite protective action of the potassium phosphate, when substituted for a part of the calcium lactate, is also not as yet to be explained; and we would carefully avoid the conclusion that rickets in these cases is due necessarily to a deficiency of potassium or of phosphorus. The quantitative relation of the inorganic ions, rather than an absolute deficiency of any one of them may be a determining factor, and it may well be that under certain conditions of diet in

which there is an unbalanced quantitative relationship of the organic as well as the inorganic foodstuffs, rickets may develop.

However, our experiments seem to demonstrate that rickets may be induced or prevented without change in either the protein or vitamine components of the diet; the presence of an adequate amount of calcium also in itself does not afford protection against the disease. These facts appear to us to be firmly established by the experiments, and lead us to question the importance attributed by some writers to the deficiency of fat-soluble A and calcium in the production of rickets. Further specific evidence bearing upon the rôle of fat-soluble A will be presented in a subsequent paper.

#### CONCLUSIONS.

1. A simple diet is presented which regularly induced rickets in young rats.
2. The substitution of 0.4 per cent secondary potassium phosphate for a small part of calcium lactate in this diet completely inhibited the development of rickets.
3. Quantitative determinations of calcium in the bodies of parallel rats showed a marked increase of calcium content in the rats receiving the added phosphate over those which developed rickets.
4. While it is thus shown by x-rays and by histological examinations and by quantitative chemical analysis that added potassium phosphate increased the assimilation and normal deposition of calcium, it may be the quantitative relationship between the inorganic ions rather than actual deficiency of any one of them which was here the determining factor in the cause or prevention of rickets. Our experiments and conclusions do not exclude the possibility of other causes of rickets than those here discussed.

We are indebted to Dr. J. M. Steiner for assistance in the x-ray examinations and to Miss F. L. MacLeod for the quantitative determinations of calcium.



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## EXPLANATION OF PLATES.

RC, resting cartilage;  $Pr_1$ , zone of growing cartilage;  $pr_2$ , zone of preparatory calcification;  $Pr_3$ , prolongation of cartilage into spongiosa;  $Sp.$ , spongiosa;  $Os$ , osteoid tissue;  $Co$ , calcified corticalis;  $CB$ , calcified bone;  $C$ , calcified cartilage;  $Po$ , periosteum;  $M$ , medullary cavity.

## PLATE 10.

FIG. 1. Rat 1750. Diet 83. Section of rib showing rachitic changes.

## PLATE 11.

FIG. 2. Rat 2605. Diet 84. Section of rib showing rachitic changes.

## PLATE 12.

FIG. 3. Rat 2611. Diet 84. Section of rib showing rachitic changes.

## PLATE 13.

FIG. 4. Rat 2606. Diet 85. Section of rib. Normal chondrocostal junction.

## PLATE 14.

FIG. 5. Rat 2612. Diet 85. Section of rib. Normal chondrocostal junction.

## PLATE 15.

FIG. 6. Rat 2623. Diet 84. Radiograph showing rachitic changes at upper epiphysis of tibia.

## PLATE 16.

FIG. 7. Rat 2624. Diet 85. Radiograph. Normal epiphyses.

## PLATE 17.

FIG. 8. Rat 2625. Maintained on Diet 13, adequate for growth. Same litter as Rats 2623 and 2624. Radiograph. Normal epiphyses.

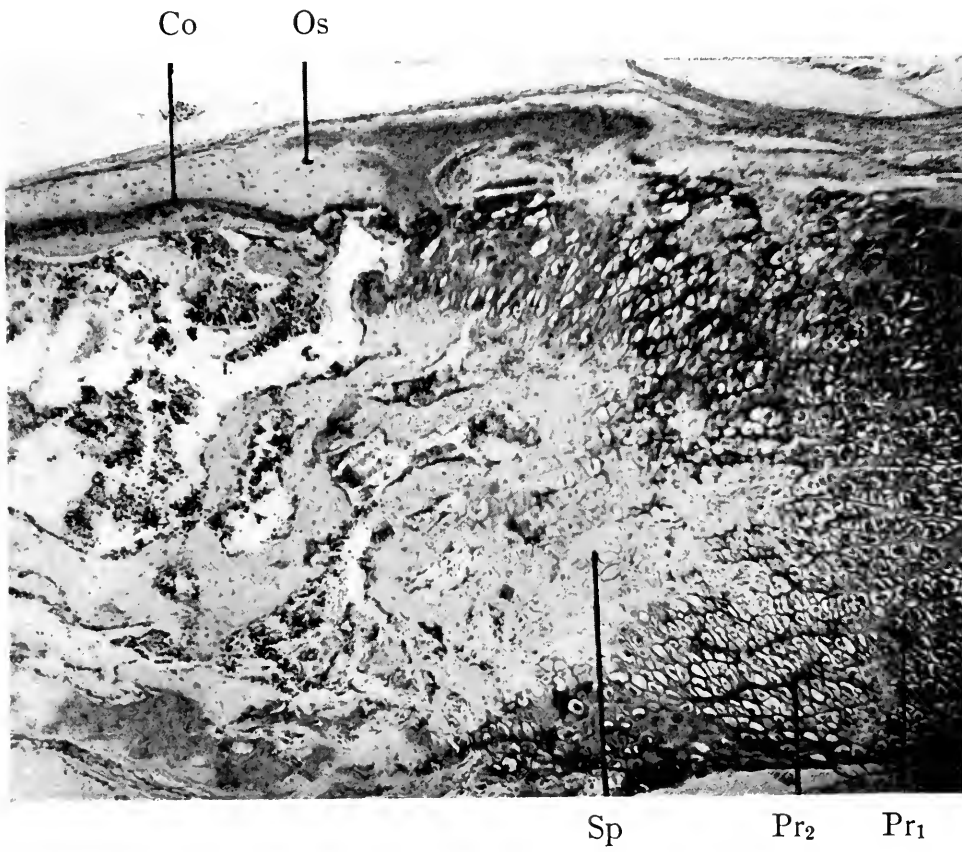
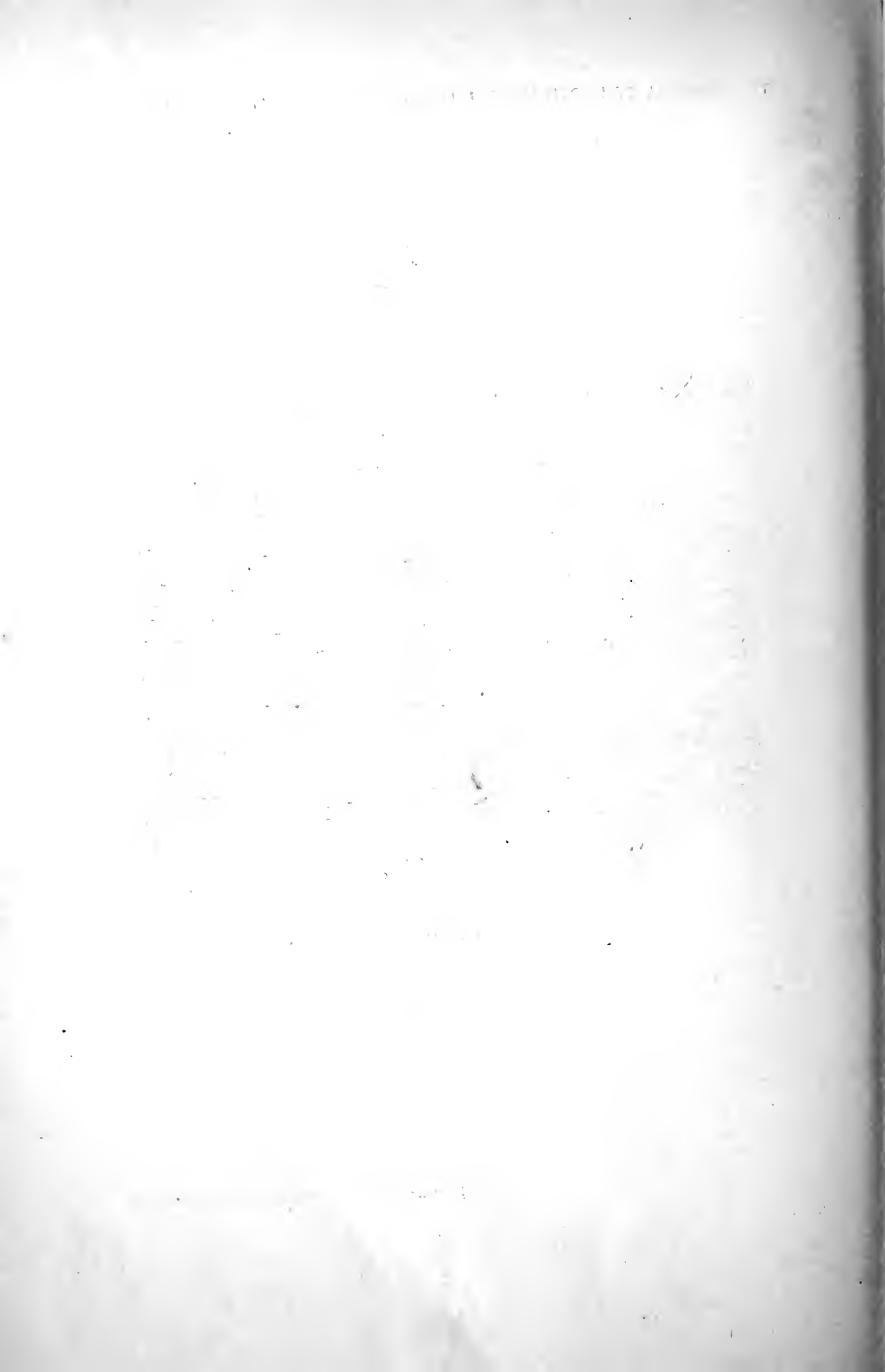


FIG. 1.

(Sherman and Pappenheimer: Experimental rickets. I.)



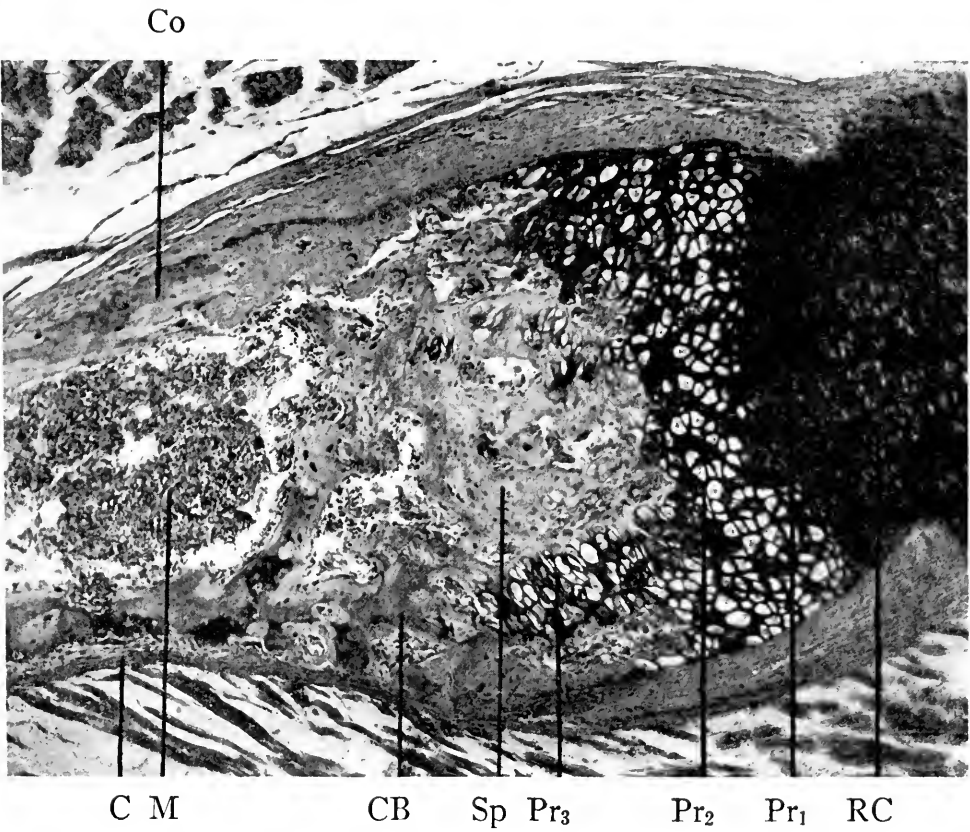
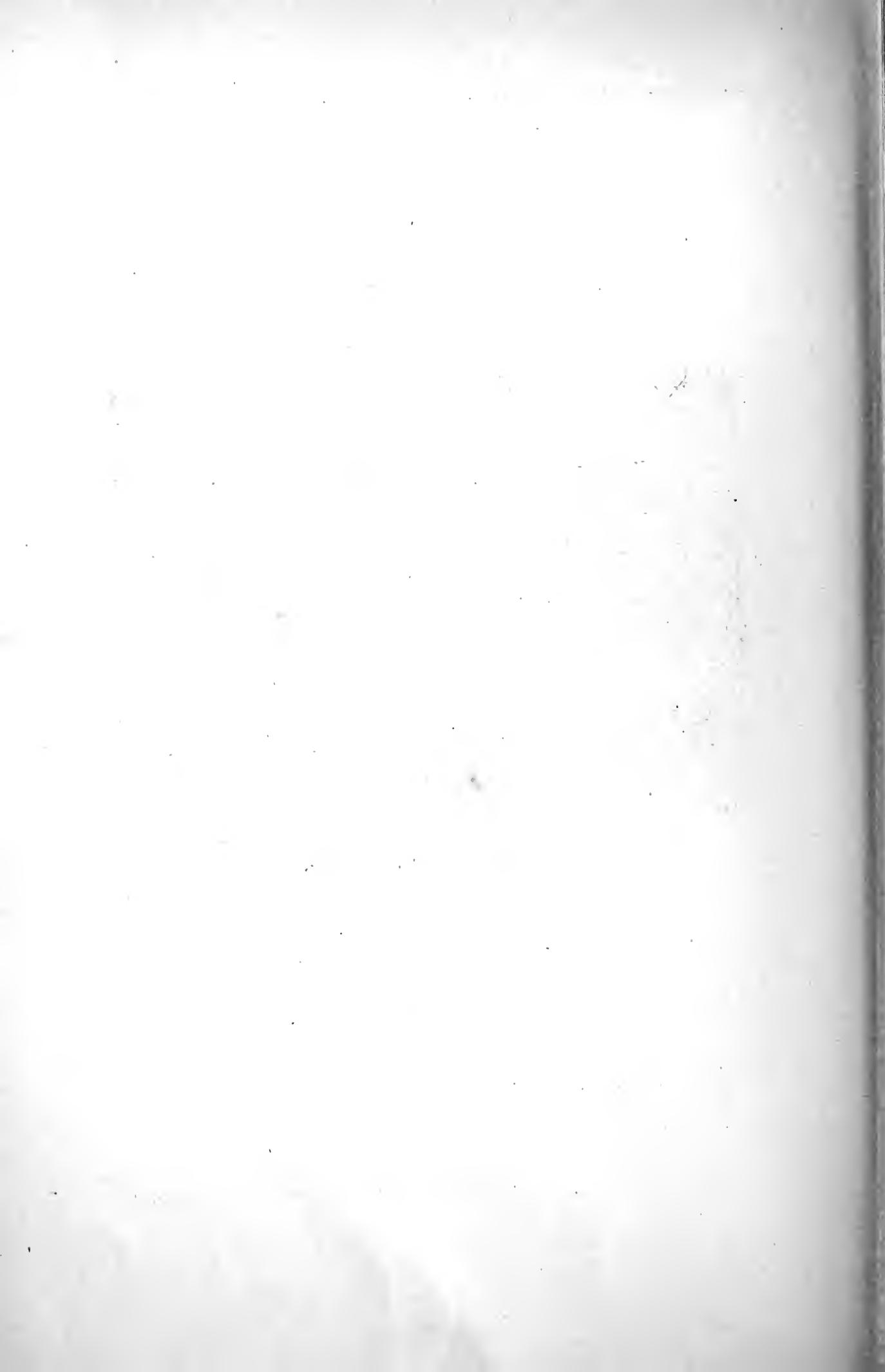


FIG. 2.

(Sherman and Pappenheimer: Experimental rickets. I.)



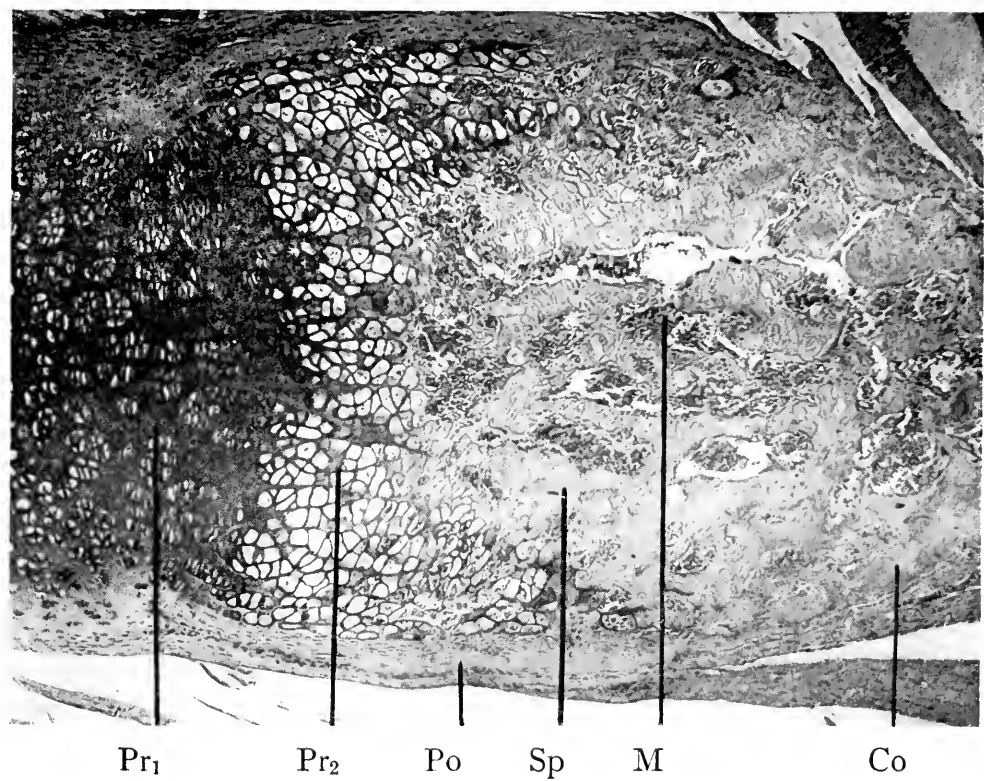


FIG. 3.

(Sherman and Pappenheimer: Experimental rickets. I.)





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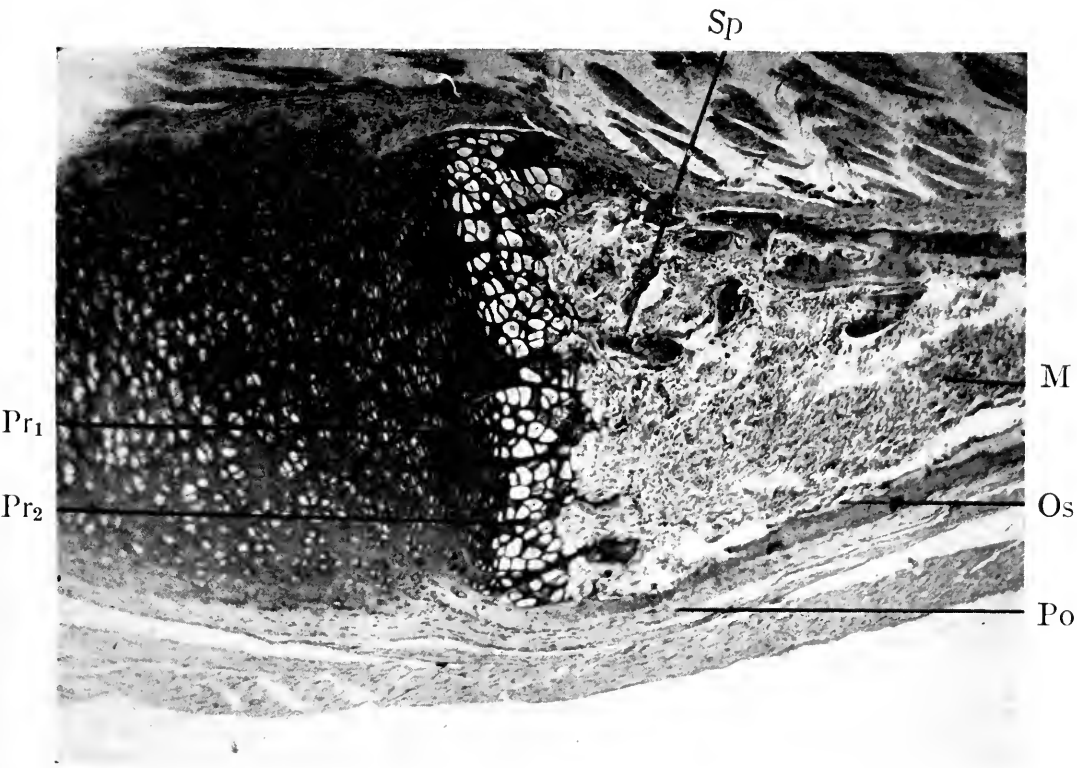


FIG. 4.

(Sherman and Pappenheimer: Experimental rickets. I.)



Pr<sub>1</sub> Pr<sub>2</sub> Sp Co M

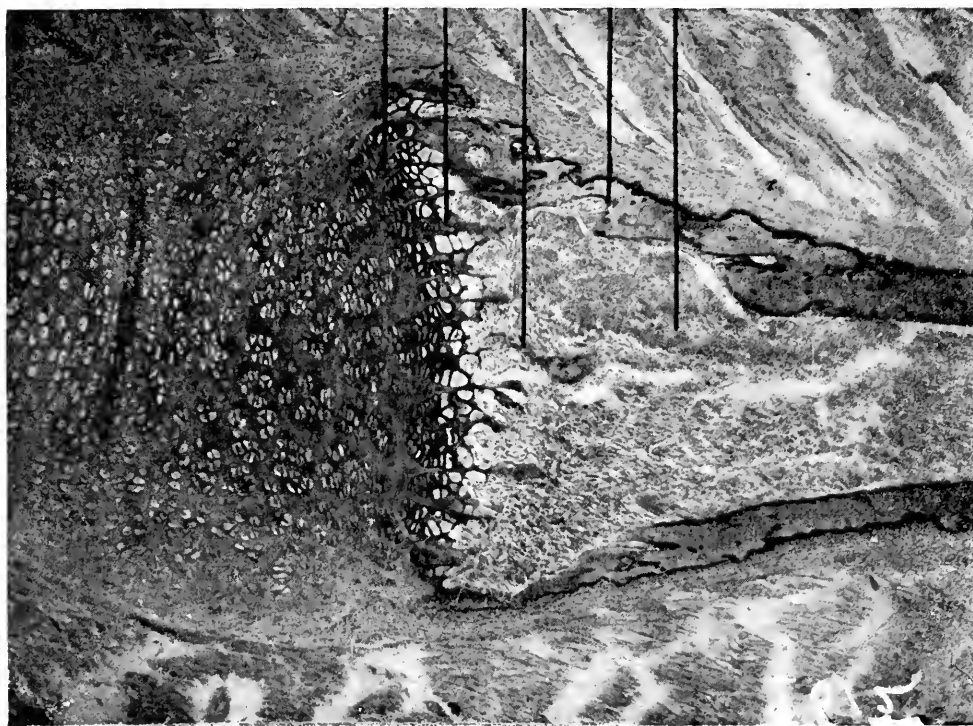


FIG. 5.

(Sherman and Pappenheimer: Experimental rickets. I.)



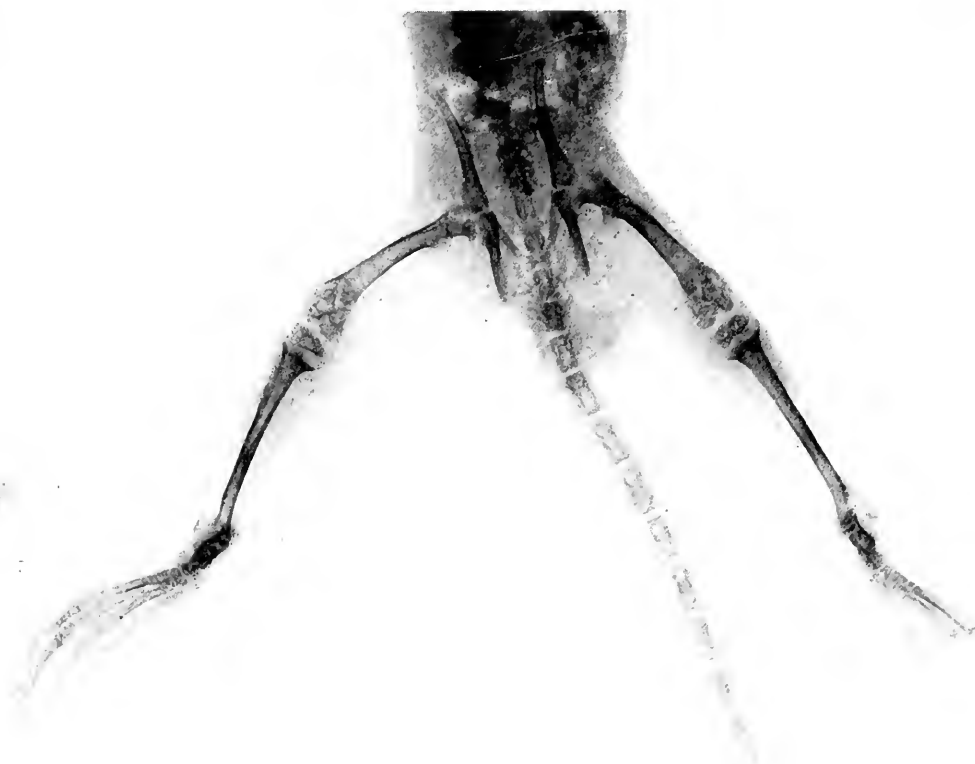


FIG. 6.

(Sherman and Pappenheimer: Experimental rickets. I.)





FIG. 7.

(Sherman and Pappenheimer: Experimental rickets. I.)

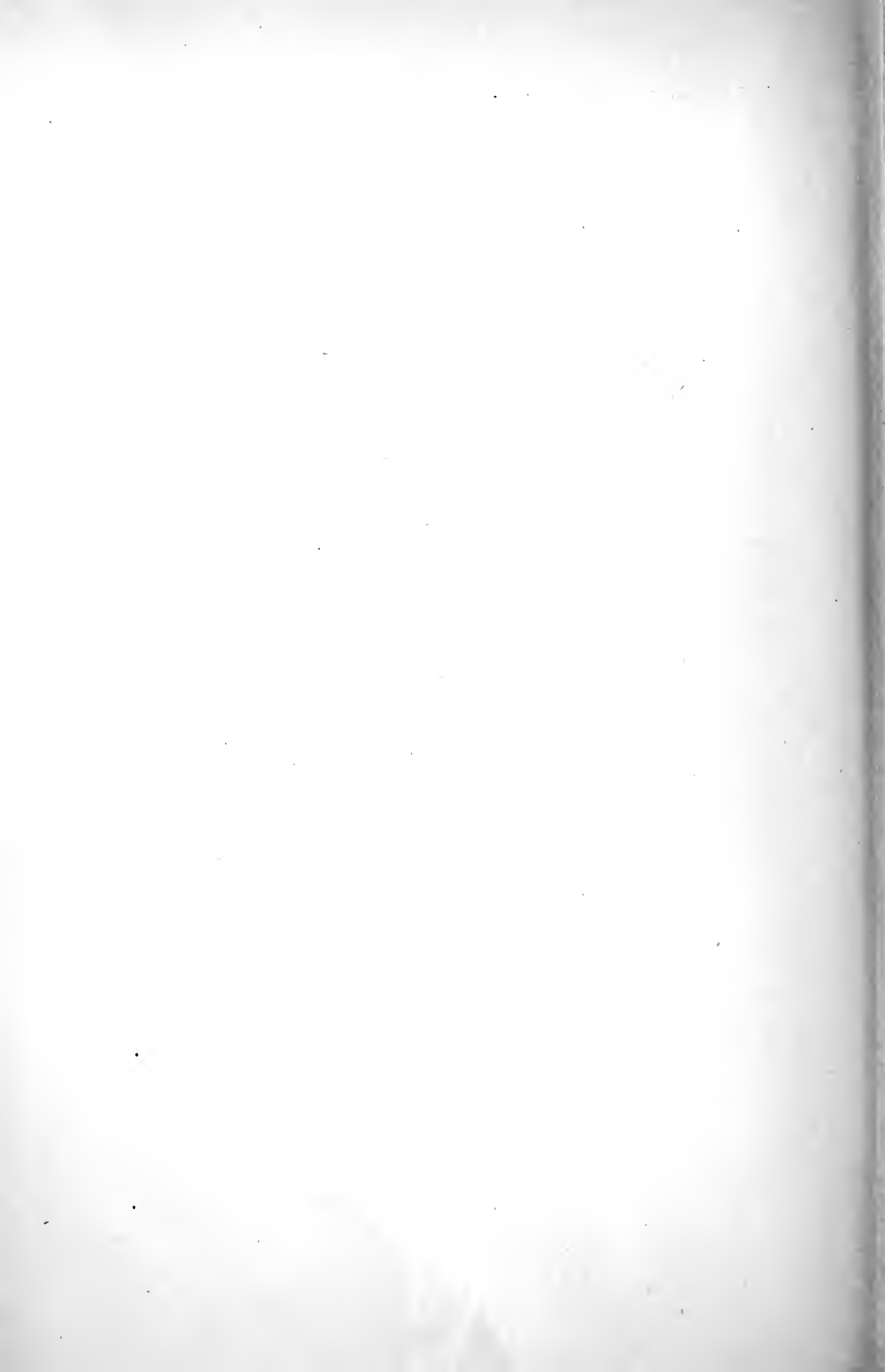






FIG. 8.

(Sherman and Pappenheimer: Experimental rickets. I.)



## STUDIES ON THE PNEUMONIC EXUDATE.

### I. EFFECT OF PRESERVATION, TEMPERATURE, DIALYSIS, AND SALT CONCENTRATION ON THE ENZYME IN THE PNEUMONIC LUNG.

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(Received for publication, March 28, 1921.)

In a previous report one of us<sup>1</sup> reported the demonstration in cellular material obtained from the pneumonic lung of a proteolytic enzyme active in eroding the surface of Löffler's blood serum at hydrogen ion concentrations of 7.3 to 6.7 and inactive at more acid concentrations. Evidence was also brought forward of the presence in the cellular material of a proteolytic enzyme splitting peptone to amino-acid nitrogen. This enzyme was operative at hydrogen ion concentrations from 8 to 4.8 but most active at 6.3 or 5.2.

In this report we present certain further observations on the conditions influencing the action of the enzyme on Löffler's blood serum.

*Maintenance of Activity of the Enzyme under Preservation.*—The enzyme capable of eroding Löffler's blood serum still remains active after preservation in the ice box mixed with chloroform and toluene for a period of 18 months.

*Effect of Temperature on the Enzyme.*—The enzyme is active at incubator temperature. It is slightly active at room temperature and inactive in the ice chest. Its activity still persists after heating at 56° or at 65°C. for 1 hour. After heating at 75°C. for 1 hour the enzyme is inactive.

*Effect of Dialysis.*—To test the penetration of a celloidin sac, the following experiment was performed. 5 cc. of cellular material obtained from the pneumonic lung were placed in a celloidin sac previously sterilized by submersion in 60 per cent alcohol for 1 hour. The cellular material was allowed to dialyze against 5 cc. of sterile normal saline solution<sup>2</sup> for 1 hour. One loopful of the saline solution

<sup>1</sup> Lord, F. T., *J. Exp. Med.*, 1919, xxx, 379.

<sup>2</sup> By normal saline in this series is meant 0.85 per cent.

outside the sac and one loopful of the cellular material from within the sac were placed on the surface of Löffler's blood serum. The saline solution showed no erosion of the medium while the cellular material from within the sac showed erosion.

*Effect of Salt Concentration on the Enzyme.*—Material obtained from a pneumonic lung was washed through gauze with normal saline solution and the washed suspension centrifuged. The heavy grayish cellular suspension thus obtained was diluted with about 3 cc. of saline solution to make about 12 cc. of total suspension. Equal parts (0.5 cc.) of this cellular suspension and solutions of sodium chloride of different normality were mixed together as follows:

Tube No.	Normality. <sup>2</sup>	Sodium chloride.	Cellular suspension.
		cc.	cc.
1	N	0.5	0.5
2	2 × N	0.5	0.5
3	4 × N	0.5	0.5
4	8 × N	0.5	0.5
5	16 × N	0.5	0.5
6	32 × N	0.5	0.5

One loopful of each mixture was placed on the surface of Löffler's blood serum and incubated over night. All showed proteolysis. Increased concentration of sodium chloride therefore does not seem to inhibit the enzymatic action of the material on Löffler's blood serum.

#### CONCLUSION.

1. The enzyme present in the pneumonic lung exudate still remains active after preservation for 18 months.
2. The enzyme is active at incubator temperature before and after heating to 65°C. for 1 hour. It is slightly active at room temperature and inactive after heating at 75°C. for 1 hour.
3. Dialysis of the enzyme is not demonstrable.
4. Activity persists when the enzyme is mixed with concentrations of sodium chloride varying from normal to thirty-two times normal.

For technical assistance in the experiments included in this and the subsequent articles of this series, we are indebted to Miss Margaret Herrick and Miss Ruth Seybolt.

## STUDIES ON THE PNEUMONIC EXUDATE.

### II. THE PRESENCE OF ENZYME AND ANTIENZYME IN THE PNEUMONIC LUNG. LOCAL FERMENT-ANTIFERMENT BALANCE.

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(Received for publication, March 28, 1921.)

It has long been evident that the exudate in lobar pneumonia disappears by absorption and the investigations of Müller<sup>1</sup> and Simon<sup>2</sup> suggest that autolysis is responsible. Simon demonstrated autodigestion in gray to a greater degree than in red hepatization. This was later confirmed by Silvestrini<sup>3</sup> and by Flexner.<sup>4</sup> The work of Opie<sup>5</sup> and others has demonstrated that the autolytic process is with probability to be regarded as due to ferments set free from the polynuclear cells.

An important relation is known to obtain between ferments set free from cells and antiferment contained in serum. Thus Opie,<sup>5</sup> in his investigation of the capacity for proteolytic digestion of pleural exudates artificially produced in dogs and rabbits, showed that the serum of an inflammatory exudate has the power of inhibiting the action of proteolytic ferments contained in the leucocytes.

Jobling, Petersen, and Eggstein<sup>6</sup> investigating the blood of patients with pneumonia found that the crisis is usually accompanied by a decrease in the serum antiferment and an increase in ferment. According to their view, ferment, probably derived from the tissue

<sup>1</sup> Müller, Fr., *Verhandl. schweizerischen Naturforschenden. Ges. Basel*, 1901, xiii, 308.

<sup>2</sup> Simon, O., *Deutsch. Arch. klin. Med.*, 1901, lxx, 604.

<sup>3</sup> Silvestrini, R., *Biochem. Centr.*, 1903, i, 713.

<sup>4</sup> Flexner, S., *Tr. Assn. Am. Phys.*, 1903, xviii, 359.

<sup>5</sup> Opie, E. L., *J. Exp. Med.*, 1905, vii, 316; 1906, viii, 410.

<sup>6</sup> Jobling, J. W., Petersen, W., and Eggstein, A. A., *J. Exp. Med.*, 1915, xxii, 568.

cells of the body in general, may be mobilized and brought to bear on the diseased organ, the active autolysis which begins at the time of crisis depending on an altered relation between the ferment-antiferment balance. As they point out, the isolation of the involved region from the general circulation favors autolysis by hindering the access of blood serum with its great concentration of antiferment. The following experiments represent an attempt to determine more clearly the source of the ferment and the application of the ferment-antiferment balance to the pneumonic exudate.

We have usually found that the purulent sputum obtained from patients in the later stages of lobar pneumonia erodes the surface of Löffler's blood serum. The exudate at autopsy from pneumonic lungs in the later stages of the disease commonly also produces erosion. Inasmuch as the culture tubes almost invariably show bacterial growth the question may be raised whether the action of living bacteria or their products may not be responsible for the erosion. In our experience, however, the bacteria found in the pneumonic lung do not alone cause erosion of the medium, and we have in one instance obtained from the pneumonic lung in the stage of purulent softening sterile material capable of eroding blood serum slants, suggesting that the action of living bacteria is not concerned in the process. Such an experience does not exclude the possibility that an enzyme derived from the dissolution of pneumococci may not be responsible.

Tests for the presence of an enzyme derived from dissolved pneumococci and capable of eroding Löffler's blood serum were performed as follows: The bacterial growth of Type I pneumococcus obtained after incubation over night of a liter flask of glucose broth was centrifuged. The sediment thus obtained was added to 8 cc. of sterile bile and incubated for 3 hours. The bile was then centrifuged at high speed. Films from the small amount of sediment showed large numbers of Gram-negative and eroded remains of pneumococci. The bile in which the pneumococci had been dissolved caused no erosion of blood serum. No evidence is presented therefore suggesting that an enzyme derived from the pneumococcus is the cause of the erosion.

The following experience indicates that a substance inhibiting proteolysis exists in early pneumonic areas and can be washed out of the exudate. Mashed material obtained from a pneumonic lung in the stage of red hepatization when placed on the surface of Löffler's blood serum failed to erode the medium. The same material after washing five or six times with normal saline solution produced erosion of the medium, indicating that the inhibiting substance was thus removed or the enzyme set free.

The following experiment shows that normal human serum in certain concentrations will inhibit the proteolytic action of the pneumonic exudate and in lower dilution still allow the proteolytic action of the exudate to take place. It furnishes strong support to the belief that the inhibiting substance removed from the lung in the previous experiment was an excess of serum containing anti ferment. Mixtures were made of cellular material obtained from the mash of a pneumonic lung in the stage of gray hepatization and normal human serum to produce varying proportions of cellular material and serum as follows:

10 cc. of the lung mash (preserved with toluene and chloroform) were ground several minutes in a mortar. 7 cc. were drawn off with a capillary pipette, placed in a centrifuge tube, and spun down for 1 minute at low speed. The supernatant fluid was pipetted off and spun down at high speed. This final sediment was washed twice with saline solution and recentrifuged at No. 7 plug on the rheostat for 7 minutes. The colorless and comparatively clear supernatant fluid was pipetted away and 0.5 cc. of "cells" remained. These were made up to the desired dilutions with saline solution. Serum was obtained from fresh human blood and dilutions were made with saline solution. Precautions to maintain sterility were used throughout. The dilutions of "cells" to make the cellular suspension and the dilutions of serum are indicated in Table I. 5 drops of the cellular suspension and 5 drops of the serum were placed in sterile tubes and kept at incubator temperature for 15 minutes. Then 1 drop of the mixtures was placed on Löffler's blood serum and the presence or absence of erosion noted after 18 to 24 hours in the incubator. No growth of bacteria was observed on the culture media.

As shown in Table I, the presence or absence of erosion depends on the proportion of cellular material to serum in the mixtures and not upon the dilution of the cellular material, since in the three groups (Tubes 1 to 4, 5 to 8, and 9 to 12) the dilution of the cellular material was the same in each tube of any one group. A fairly definite relation obtains between the proportion of cellular material to serum and the production of erosion. No erosion was noted when the ratio was less than one part of cellular material to four parts

TABLE I.

*Ferment-Antiferment Balance in the Erosion of Löffler's Blood Serum.*

Tube No.	Dilution.		Amount of cellular suspension.	Dilution.		Amount of serum.	Final dilution.		Ratio of "cells" to serum.	Erosion.
	"Cells."	Saline solution.		Serum.	Saline solution.		"Cells."	Serum.		
			gll.			gll.				
1	1 part.	4 parts.	5	1 part.	0	5	1:10	1:2	1:5.0	0
2	1 "	4 "	5	1 "	1 part.	5	1:10	1:4	1:2.5	+
3	1 "	4 "	5	1 "	4 parts.	5	1:10	1:10	1:1.0	++
4	1 "	4 "	5	1 "	6.5 "	5	1:10	1:15	1:0.67	+++
5	1 "	9 "	5	1 "	0	5	1:20	1:2	1:10.0	0
5	1 "	9 "	5	1 "	1 part.	5	1:20	1:4	1:5.0	0
7	1 "	9 "	5	1 "	4 parts.	5	1:20	1:10	1:2.0	+
8	1 "	9 "	5	1 "	6.5 "	5	1:20	1:15	1:1.33	++
9	1 "	19 "	5	1 "	0	5	1:40	1:2	1:20.0	0
10	1 "	19 "	5	1 "	1 part.	5	1:40	1:4	1:10.0	0
11	1 "	19 "	5	1 "	4 parts.	5	1:40	1:10	1:4.0	0
12	1 "	19 "	5	1 "	6.5 "	5	1:40	1:15	1:2.66	+(?)

of serum. Questionable erosion resulted when there was one part of cells to 2.66 parts of serum, and enzymatic action was demonstrable when the cellular material exceeded this amount in the ratio.

#### DISCUSSION.

The autolysis of the pneumonic exudate may be regarded as a local chemical process capable of its peculiar evolution because of the isolation of the alveolar exudate from the general circulation by the limiting alveolar wall. The disintegration of the cellular



alveolar exudate is the chief source of ferments and the activity of the ferments depends on the amount of serum in the affected region. As the inflammatory process advances the cellular elements become more numerous and the serum less abundant. The cellular ferments increase with the continued disintegration of the cells and the inhibiting action of the serum ceases in the alveoli, allowing proteolysis to take place. The experiment cited above suggests that antienzymatic action of the serum terminates when the cells reach a greater concentration than one part of cells to approximately three parts of serum. The framework of the lung may be assumed to be supplied with sufficient blood to maintain the necessary excess of anti-ferment. By such a mechanism as this the lung framework is spared permitting resolution of the exudate and on the part of the lung restoration to normal. It is not surprising also that at times the mechanism should fail in consequence of an improper local ferment-antiferment balance. Too great an impairment of the circulation and an excess of ferment are probably concerned in the disintegration of the pulmonary framework in postpneumonic abscess. Too free a supply of blood with its content of antiferment may be regarded as of importance in delayed resolution.

#### CONCLUSIONS.

The purulent sputum obtained during life and the exudate at autopsy from the later stages of lobar pneumonia commonly erode the surface of Löffler's blood serum. Cellular material obtained from the pneumonic lung in an early stage of lobar pneumonia failed to erode the surface until washed with normal saline solution. Mixtures of washed pneumonic cellular material and normal human serum fail to erode Löffler's blood serum when the amount of cellular material is less than one part of cells to approximately three parts of serum. Erosion occurs when the cellular material exceeds this amount in the ratio.



## STUDIES ON THE PNEUMONIC EXUDATE.

### III. THE PRESENCE IN THE PNEUMONIC EXUDATE OF A LARGE AMOUNT OF SPECIFIC ANTIGEN.

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It is well known that the pneumococcus as well as certain other organisms produces in the media in which it grows a substance which may be precipitated by the homologous immune serum. This specific precipitation occurs in the filtrates of bouillon cultures, in salt solution, and bile extracts. Dochez and Avery<sup>1</sup> have demonstrated such a substance in the cell-free fluid of young cultures and in the blood and urine of experimentally infected rabbits and in human beings. Blake and others<sup>2</sup> showed that a specific precipitation is present in the peritoneal exudate of infected animals. These observations and also Krumwiede and Valentine's finding<sup>3</sup> of soluble antigen in the sputum of patients with lobar pneumonia, made it highly probable that antigen would be readily demonstrable in the pneumonic lung. As we have seen no publication bearing on this matter we present the following simple experiments to determine the presence or absence in the pneumonic lung of antigenic substances capable of causing a specific precipitin reaction with antipneumococcus horse serum.

*Soluble Antigen in the Pneumonic Exudate. Detection by the Precipitin Method.*—Blocks of a pneumonic lung (Type I pneumococcus)

<sup>1</sup> Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.

<sup>2</sup> Blake, F. G., *J. Exp. Med.*, 1917, xxvi, 67. Avery, O. T., Chickering, H. T., Cole, R., and Dochez, A. R., Acute lobar pneumonia. Prevention and serum treatment, Monograph of The Rockefeller Institute for Medical Research, No. 7, New York, 1917, 27.

<sup>3</sup> Krumwiede, C., and Valentine, E., *J. Am. Med. Assn.*, 1918, lxx, 513.

were washed in normal saline solution, passed through a meat cutter, and the resulting mash was preserved in a preserve jar with toluene and chloroform. A part of this material was centrifuged and the supernatant fluid diluted with an equal volume of normal saline solution. This fluid when mixed with diagnostic horse serum showed precipitation in the tubes containing Type I antipneumococcus serum. No precipitation was noted in the tubes containing Type II and III sera.

Another portion of the same lung mash was centrifuged and the resulting supernatant fluid was pipetted off and diluted with an equal volume of normal saline solution from which increasing dilutions were further made. Five tubes were then prepared each con-

TABLE I.

*Precipitation Test for Specific Antigen with Increasing Dilution of Exudate.*

Tube No.	Type I serum.	Pneumonic fluid diluted with equal quantity of saline solution.	Incubation at 37°C. for 30 min.	Final dilution.		Precipitation.
				Serum.	Pneumonic fluid.	
	cc.					
1	0.4	0.4 cc. of dilution 1:10.		1:2	1:40	++++
2	0.4	0.4 " " " 1:20.		1:2	1:80	+++
3	0.4	0.4 " " " 1:40.		1:2	1:160	++
4	0.4	0.4 " " " 1:80.		1:2	1:320	+
5	0.4	0.4 " " " 1:160.		1:2	1:640	0

taining 0.4 cc. of antipneumococcus horse serum and 0.4 cc. of increasing dilutions of the fluid obtained from the pneumonic lung, as shown in Table I. In making the test a constant amount of antiserum (precipitin) was thus mixed with a progressively diminishing amount of antigen (pneumonic fluid) in the same volume. The tubes were incubated at 37°C. for 30 minutes. The precipitin tests were positive in as high a dilution as 1:320 of the pneumonic fluid.

The reaction is doubtless due to the presence in the pneumonic lung of a large amount of Type I pneumococcus extract derived from the dissolution of the organism. Tests similarly performed with other pneumonic exudates show that infection with Type I,

II, or III pneumococcus can be determined by a precipitin reaction when the exudate is mixed with the homologous serum. The reaction appears to be specific. No group reaction has been observed in these tests.

Similar tests were made maintaining the concentration of the pneumonic fluid constant and increasing the dilution of the serum, as shown in Table II. After incubation at 37°C. for 30 minutes precipitin tests were positive in as high a dilution as 1:20 of the serum.

TABLE II.

*Precipitation Test for Specific Antigen with Increasing Dilution of Serum.*

Tube No.	Type I serum.	Pneumonic fluid diluted with equal quantity of saline solution.		Final dilution.		Precipitation.
				Serum.	Pneumonic fluid.	
		cc.	Incubation at 37°C. for 30 min.			
1	0.4 cc.	0.4		1:2	1:4	++++++
2	0.4 " of dilution 1:10.	0.4		1:20	1:4	++
3	0.4 " " " 1:20.	0.4		1:40	1:4	0
4	0.4 " " " 1:40.	0.4		1:80	1:4	0
5	0.4 " " " 1:80.	0.4		1:160	1:4	0
6	0.4 " " " 1:160.	0.4		1:320	1:4	0
7	0.4 " " " 1:320.	0.4		1:640	1:4	0

## CONCLUSION.

In lobar pneumonia due to the fixed types of pneumococci a specific precipitin reaction is obtained when the pneumonic exudate is mixed with the homologous antipneumococcic serum.



## STUDIES ON THE PNEUMONIC EXUDATE.

### IV. THE PRESENCE IN THE PNEUMONIC LUNG OF A SOLUBLE SUBSTANCE INHIBITING AGGLUTINATION BY THE HOMOLOGOUS SERUM.

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Besançon and Griffon,<sup>1</sup> Chickering,<sup>2</sup> Blake,<sup>3</sup> and others have shown that agglutinins appear in the blood of lobar pneumonia patients during the course of the disease or during convalescence. In Blake's series, agglutinins for the homologous pneumococcus appeared in the blood in all patients who recovered, just before, during, or after crisis or lysis. Their concentration increased rapidly at the time of recovery and remained elevated during convalescence in the milder cases and showed a tendency to fall off in the more severe cases. Reference to his charts shows that in one case the amount of agglutinin, as measured by the highest dilution in which a positive test occurred, reached such a concentration that the homologous organism was agglutinated by a dilution of 1:32 of the patient's serum and that in two other cases it reached 1:16. Fatal cases failed to develop demonstrable agglutinins in the blood.

Cole<sup>4</sup> has noted that empyema fluids due to pneumococcus infection contain large amounts of soluble substances which have the property of neutralizing pneumococcus antibodies. For the purpose of his investigation, as indicated by the protocol of one of his experiments, 0.4 cc. of empyema fluid due to Type I pneumococcus, cleared by centrifuging, and mixed with an equal volume of an increasing dilution of antipneumococcus serum, was tested for its power to cause agglutination of Type I pneumococcus. The admixture of pneumonic

<sup>1</sup> Besançon, F., and Griffon, V., *Compt. rend. Soc. biol.*, 1897, xlix, 551, 579.

<sup>2</sup> Chickering, H. T., *J. Exp. Med.*, 1914, xx, 599.

<sup>3</sup> Blake, F. G., *Arch. Int. Med.*, 1918, xxi, 779.

<sup>4</sup> Cole, R., *J. Exp. Med.*, 1917, xxvi, 453.

fluid with the homologous antipneumococcus serum in our hands always gave rise to a precipitate which we have had to remove by the centrifuge.

*Agglutinins in Pneumonic Lung Exudate.*—In order to determine to what degree specific agglutinins for the homologous organism may be present in the pneumonic lung, the following experiment was performed with the macroscopic method.

The lung mash from a case of Type I pneumonia was centrifuged and increasing dilutions of the supernatant fluid were transferred to small test-tubes, each containing 5 drops of a suspension of Type I pneumococcus, as shown in Table I. After 2 hours incubation agglutination was noted in a dilution of 1:2 of the exudate, questionable agglutination at 1:10, and none at 1:20. A control series con-

TABLE I.  
*Tests for Agglutinins in Type I Pneumococcic Exudate.*

Tube No.	Suspension of Pneumococcus Type I.	Lung exudate.		Final dilution of lung exudate.	Agglutination.*
		Amount.	Dilution.		
	<i>gtt.</i>	<i>gtt.</i>			
1	5	5	Undiluted.	1:2	+
2	5	5	1:5	1:10	?
3	5	5	1:10	1:20	0

\* Observation after incubation for 2 hours and in ice box over night.

taining the same strain of Type I pneumococcus and homologous antipneumococcus horse serum showed agglutination in a dilution as high as 1:80 of the serum.

The lung mash from a second case of fatal Type I lobar pneumonia was similarly tested. The clear fluid was mixed in increasing dilution with normal saline solution. To each tube were added 2 drops of a suspension of Type I pneumococcus. No agglutination of the organisms was noted at any dilution, the lowest being 1:4 of the lung fluid. Antipneumococcus horse serum agglutinated the same strain in as high a dilution as 1:40 of the serum.

In a case of Type III pneumonia no agglutination was observed at any dilution, the strongest used being 1:2 of the exudate. In tests simultaneously performed Type III antipneumococcus horse



TABLE II.

*Agglutination Titer with Normal Saline Solution.*

Tube No.	Serum dilution.	Agglutination.
1	1:10	+
2	1:20	++
3	1:40	++++
4	1:80	++
5	1:160	+
6	1:320	0

0.5 cc. of increasing dilutions of Type III antipneumococcus serum + 0.5 cc. of normal saline solution + 3 drops of Type III pneumococcus suspension.

TABLE III.

*Agglutination Titer with Normal Saline Solution and Chloroform.*

Tube No.	Serum dilution.	Agglutination.
1	1:10	+
2	1:20	++
3	1:40	++++
4	1:80	++
5	1:160	0
6	1:320	0

0.5 cc. of increasing dilutions of Type III antipneumococcus serum + 0.5 cc. of normal saline solution + 3 drops of chloroform + 3 drops of Type III pneumococcus.

TABLE IV.

*Agglutination Titer with Normal Saline Solution and Toluene.*

Tube No.	Serum dilution.	Agglutination.
1	1:10	+
2	1:20	++
3	1:40	++++
4	1:80	+
5	1:160	0

0.5 cc. of increasing dilutions of Type III antipneumococcus serum + 0.5 cc. of normal saline solution + 0.5 cc. of toluene + 3 drops of Type III pneumococcus.

serum agglutinated the same strain of pneumococcus in a dilution as high as 1:40 of the serum.

To exclude the possibility that the method of preservation with toluene and chloroform might influence the result, the agglutination titer of Type III antipneumococcus serum against the homologous organism was determined and found not to vary more than might be expected from some slight but indeterminable added dilution of the serum when the serum was mixed with (1) normal saline solution (Table II), (2) saline solution and chloroform (Table III), (3) saline solution and toluene (Table IV), and (4) saline solution, chloroform, and toluene (Table V).

TABLE V.

*Agglutination Titer with Normal Saline Solution, Toluene, and Chloroform.*

Tube No.	Serum dilution.	Agglutination.
1	1:10	++++
2	1:20	++++
3	1:40	++++
4	1:80	0
5	1:160	0

0.5 cc. of increasing dilutions of Type III antipneumococcus serum + 0.5 cc. of normal saline solution + 0.5 cc. of toluene + 3 drops of chloroform + 3 drops of Type III pneumococcus.

Our previous experiments indicate that there are only very small amounts of, or no agglutinins for the homologous pneumococcus in the pneumonic exudate, although it is still possible that agglutinins may be present and undemonstrable in consequence of neutralization by the large amount of dissolved bacterial extract in the exudate. There is, in fact, evidence that when antipneumococcus serum is mixed with the homologous exudate a considerable amount of agglutinin is removed from the serum, as indicated by the experiment illustrated in Table VI.

In a series of tubes numbered A<sub>1</sub>, B<sub>1</sub>, and C<sub>1</sub>, after mixing varying dilutions of homologous antipneumococcus serum and Type I pneumonic exudate diluted with an equal volume of saline solution, the resulting precipitate was removed by means of the centrifuge. 10

drops of the clear supernatant fluid were then removed from each tube to fresh tubes and to each 2 drops of a suspension of Type I pneumococcus were added. As indicated in Table VI agglutination occurred only in Tube A<sub>1</sub> which contained a dilution of 1:2 of serum and 1:4 of pneumonic fluid.

When, however, to a second set of tubes numbered A<sub>2</sub>, B<sub>2</sub>, and C<sub>2</sub>, containing 0.4 cc. of increasing dilutions of the same lot of Type

TABLE VI.

*Removal of Agglutinin from Antipneumococcic Serum by Homologous Pneumococcus Exudate.*

Tube No.	Type I serum.		Normal saline solution.	Pneumonic fluid + equal volume of normal saline solution.		Final dilution.		Suspension of Pneumococci Type I.	Agglutination.*
	Amount.	Dilution.		Amount.	Dilution.	Serum.	Pneumonic fluid.		
	cc.		cc.	cc.				glt.	
A <sub>1</sub>	0.4	Undiluted.		0.4	Undiluted.†	1:2	1:4	2	+
B <sub>1</sub>	0.4	1:10		0.4	" †	1:20	1:4	2	0
C <sub>1</sub>	0.4	1:20		0.4	" †	1:40	1:4	2	0
A <sub>2</sub>	0.4	1:10	0.4			1:20		2	+
B <sub>2</sub>	0.4	1:20	0.4			1:40		2	+
C <sub>2</sub>	0.4	1:40	0.4			1:80		2	0
A <sub>3</sub>			0.4	0.4	Undiluted.		1:4	2	0
B <sub>3</sub>			0.4	0.4	1:10		1:40	2	0
C <sub>3</sub>			0.4	0.4	1:20		1:80	2	0

\* The plus signs indicate the presence of agglutination, without regard to its degree.

† Centrifuged to remove precipitate.

I antipneumococcus serum and prepared at the same time, 0.4 cc. of normal saline solution were added in place of the pneumonic exudate and 2 drops of the same suspension of Type I pneumococcus, agglutination took place in as high a dilution as 1:20 and 1:40 of the serum. There are; therefore, substances in the exudate which inhibit agglutination or the agglutinins are removed by the precipitation.

As a control, in Tubes A<sub>3</sub>, B<sub>3</sub>, and C<sub>3</sub>, 2 drops of the same suspension of Type I pneumococcus were added to increasing dilutions of the pneumonic fluid plus an equal volume of normal saline solution. No agglutination took place at any dilution, the highest strength of the pneumonic fluid used being 1:4. The agglutination of the organisms in the mixture of antipneumococcus serum and pneumonic exudate (Tube A<sub>1</sub>) is in consequence to be ascribed to the agglutinins in the serum rather than any in the pneumonic exudate.

#### CONCLUSIONS.

Specific agglutinins for the homologous pneumococcus are lacking or present only in small amount in the pneumonic exudates due to the fixed types of pneumococci. Suspensions of fixed types of pneumococci in the supernatant fluid obtained after centrifuging the mash of the pneumonic lung give positive agglutination tests in dilutions not higher than equal parts of suspension and supernatant fluid (1:2). The pneumonic lung contains a soluble substance inhibiting agglutination of the fixed types of pneumococci by the homologous antipneumococcus serum.

## STUDIES ON COMPLEMENT FIXATION.

### I. THE RATE OF FIXATION OF COMPLEMENT AT DIFFERENT TEMPERATURES.

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(Received for publication, April 1, 1921.)

#### INTRODUCTION.

A more complete understanding of the complement fixation phenomenon will perhaps ultimately be achieved by the employment of purified proteins as antigens. The mass of accumulated studies of this phenomenon with organ extracts as antigens may be said to apply largely to the Wassermann test. For, it is questionable whether a principle derived with a non-specific organ extract antigen can apply in all cases to specific antigen-antibody complexes. These Wassermann studies might indeed explain the limited application of the complement fixation test to the diagnosis of diseases other than syphilis and to the identification of unknown organisms.

The antigen, however, is not the only element in which the Wassermann test varies from specific complement fixation tests. Recent studies reported from this laboratory (1) indicate that the behavior of the so called syphilitic complement-fixing antibody towards heat is markedly different from the behavior towards this agent of the complement-fixing antibody found in rabbits after immunization with purified proteins. The former is completely destroyed at temperatures ranging between 62° and 65°C., while the thermal destructive point of the latter is in the neighborhood of 80°C.

The complement fixation reaction, furthermore, is so complex, that the reduction of even one of its ingredients to the simplest elements ought to help in its interpretation. And although chemically the protein molecule is a huge structure indeed, biologically it stands out

as the simplest possible element capable of producing complement-fixing antibodies (2).

These considerations led us to undertake a series of investigations of the complement fixation phenomenon with purified proteins as antigens. These studies are still being continued and are being extended also to organ extracts with syphilitic sera as well as bacterial antigens with their specific antisera with a view of finding whether the principles developed by means of protein antigens are applicable also to practical tests.

The factors governing the fixation of complement were chosen as the subject of the first paper of this series, because of their fundamental importance in all complement fixation studies. Investigations on the effects of temperature and time on the fixation of complement have heretofore been restricted largely to the Wassermann test. The literature covering this field will be presented in a forthcoming paper in which the rate of fixation of complement in the Wassermann test will be considered. In this connection, the work of Dean (3) needs quoting inasmuch as in his studies on the effect of temperature on the fixation of complement, he employed a specific antigen—horse serum. This investigator employed a 1 hour fixation period at temperatures of 0°, 17°, and 37° C., and found uniformly more complement fixed at 0° C. than at 17° C. and more at 17° C. than at 37° C. This work is of especial interest in view of the fact that in accordance with the prevailing opinion (4, 5) the phenomenon of fixation of complement takes place far more readily at water bath than at colder temperatures.

#### EXPERIMENTAL.

The plan of these studies was, first to establish the presence of specific complement-fixing antibodies in the sera of rabbits previously immunized with purified proteins; second, to determine by means of complement fixation tests with the immune sera and specific protein antigens, the rate of fixation of complement, and to what extent this rate is affected by different temperatures.

The antigens employed were edestin, obtained from hemp-seed, and phaseolin, obtained from the kidney bean.<sup>1</sup> Three fixation tem-

<sup>1</sup> These were kindly furnished by Dr. Thomas B. Osborne of the Connecticut Agricultural Experiment Station.

peratures were adhered to: ice box (8–12°C.), room (18–23°C.), and water bath (37.5°C.). The degree of fixation was determined every 15 minutes during the 1st hour, at half hour periods during the 2nd hour, followed by determinations every hour until 5 or 6 hours had passed.

### *Methods of Immunization.*

Three different methods of immunization were resorted to in order to vary the antibody content in the rabbit sera. Rabbit A received five intravenous injections of edestin at 48 hour intervals. The quantities of protein injected were 50, 75, 100, 125, and 150 mg. respectively. Rabbit B received five intraperitoneal injections of phaseolin at 48 hour intervals, the quantities being 100, 150, 200, 250, and 300 mg. respectively. Rabbit C received three intraperitoneal injections of phaseolin at 24 hour intervals, the quantities being 100, 150, and 200 mg. respectively.

Both edestin and phaseolin are insoluble in water but soluble in weak alkaline solutions. This necessitated the addition of a few drops of 0.1N sodium hydroxide to the proteins in order to get them in solution. This was done in each case just before the injection.

### *Procedure of the Complement Fixation Test.*

The sheep cell system was employed. All ingredients entering into the test were used in 0.1 cc. quantities except the immune rabbit serum which was graded from 0.01 to 0.0001 cc. Two units of amboceptor, two units of complement, and from two to ten units of antigen were employed.

*Sheep Cells.*—Sheep were bled from the jugular vein into sterile bottles containing glass beads. After defibrination by shaking, the blood was washed four times with saline solution. The final centrifugation was carried out for 14 minutes at 1,500 revolutions per minute. 5 per cent was the strength of the standard suspension.

*Hemolysin.*—This was prepared in the usual manner by injecting rabbits with washed sheep cells. The unit of hemolysin was determined by titrating a series of dilutions of hemolysin serum with 0.1 cc. quantities of 1:10 pooled guinea pig complements and 0.1 cc. quantities of the standard sheep cell suspension and reading the unit

after 15 minutes incubation in the water bath (6). The serum dilution aimed at was one which contained two units of hemolysin in 0.1 cc.

*Complement.*—Large sized guinea pigs were bled directly from the heart. The serum was drawn after permitting the blood to remain for about 20 hours in the ice box. Pooled serum from four to five guinea pigs was used with each experiment. The unit of complement was obtained by titrating gradations of 0.1 to 0.01 cc. of both single and pooled complements with two units (0.1 cc.) of amboceptor and 0.1 cc. quantities of sheep cells. The unit in this case also was read after 15 minutes incubation in the water bath.

*Protein antigens.*—These were prepared by weighing 10 mg. of the protein and dissolving it in 10 cc. of 0.001N sodium hydroxide solution to which was added 0.05 cc. of 0.1N sodium hydroxide. The alkali used, represents approximately the smallest quantity necessary to get the proteins into solution. 1 cc. of this antigen solution was mixed with 9 cc. of salt solution and 0.1 cc. of this final solution, corresponding to 0.01 mg. of antigen, was used in the tests. Antigenic titrations of this solution with 0.01 cc. of specific immune serum and two units of complement showed, after primary incubation for 4 hours in the ice box, complete fixation of complement with 0.02 cc. (0.002 mg. of protein) and partial fixation with 0.01 cc. (0.001 mg. of protein). Tests for anticomplementary and hemolytic properties of these protein antigens showed that neither possessed these properties with five times the quantity (0.5 cc.) of antigen used in the tests.

*Immune Serum.*—The immune serum was obtained in each case by bleeding the rabbits from the marginal ear vein and separating the serum after permitting the blood to remain for several hours in the ice box. Both the edestin and phaseolin rabbits showed the presence of specific complement-fixing antibodies about 10 days after the last injection of protein. The sera were inactivated in each case for 30 minutes at 56° C. before using, and were employed in the following dilutions in the tests: 0.01, 0.007, 0.004, 0.003, 0.002, 0.001, 0.0005, 0.0003, 0.0001 cc.

*Complement Fixation Tests.*—The tests were carried out in the usual manner, regular Wassermann tubes being employed with the various gradations of serum, 0.1 cc. (0.01 mg.) of the protein antigen, 0.1 cc. (two units) of complement, and 0.1 cc. of saline solution. After



a given fixation period 0.1 cc. of the standard sheep cell suspension and 0.1 cc. of hemolysin (two units) were added and incubated in the water bath at 37.5° C. for about 15 minutes when the serum and antigen controls would be completely hemolyzed. All readings were made after keeping the tubes in the ice box over night. The temperatures and periods of fixation were the only variables in each test.

*Effect of Temperature on the Rate of Fixation of Complement.*

Contrary to the accepted views that the fixation of complement takes place far more rapidly at water bath than at a lower temperature, preliminary experiments with the rabbit sera and specific protein antigens indicated no marked difference in the degree of fixation between water bath (37.5° C.) and ice box (8–12°C.) fixation temperatures. This indeed is the reason why no attempt was made in these studies to use precise temperatures in the case of ice box and room temperature fixation. Table I gives an outline of one of these experiments with edestin immune serum. 15 minutes was the time interval chosen for fixation during the 1st hour, and multiples of 15 minutes for the succeeding hours—up to 5 or 6. It is evident from this table that the degree of fixation as measured by the increase in number of positive signs is approximately the same whether ice-box or water bath fixation is employed. There is indeed a tendency for stronger fixation in the ice box than in the water bath.

The fixation experiments at water bath and room temperatures were not extended beyond 2 hours in view of the marked deterioration of complement which takes place after prolonged exposure at these temperatures.

Text-fig. 1 shows the nature of the curve with the degree of fixation as determined by the increase in number of positive signs as the ordinate and time of fixation as the abscissa.

Table II and Text-fig. 2 represent a similar experiment with phaseolin immune serum.

Tables III and IV and Text-figs. 3 and 4 represent fixation experiments carried out at water bath, room, and ice box temperatures. The tendency for somewhat stronger fixation in the ice box is shown by

these experiments; also the fixation at room temperature approximates more closely fixation in the ice box than that in the water bath.

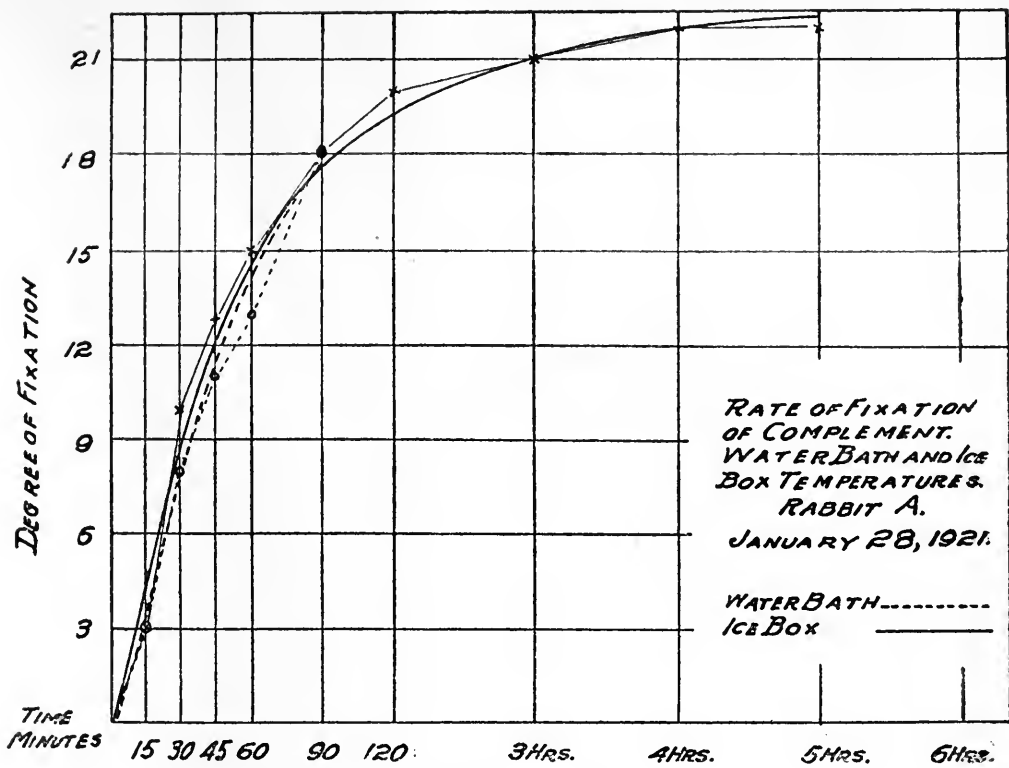
Text-figs. 5 and 6 show a marked similarity with the previous experiments and indicate to what extent the curves of fixation approximate

TABLE I.

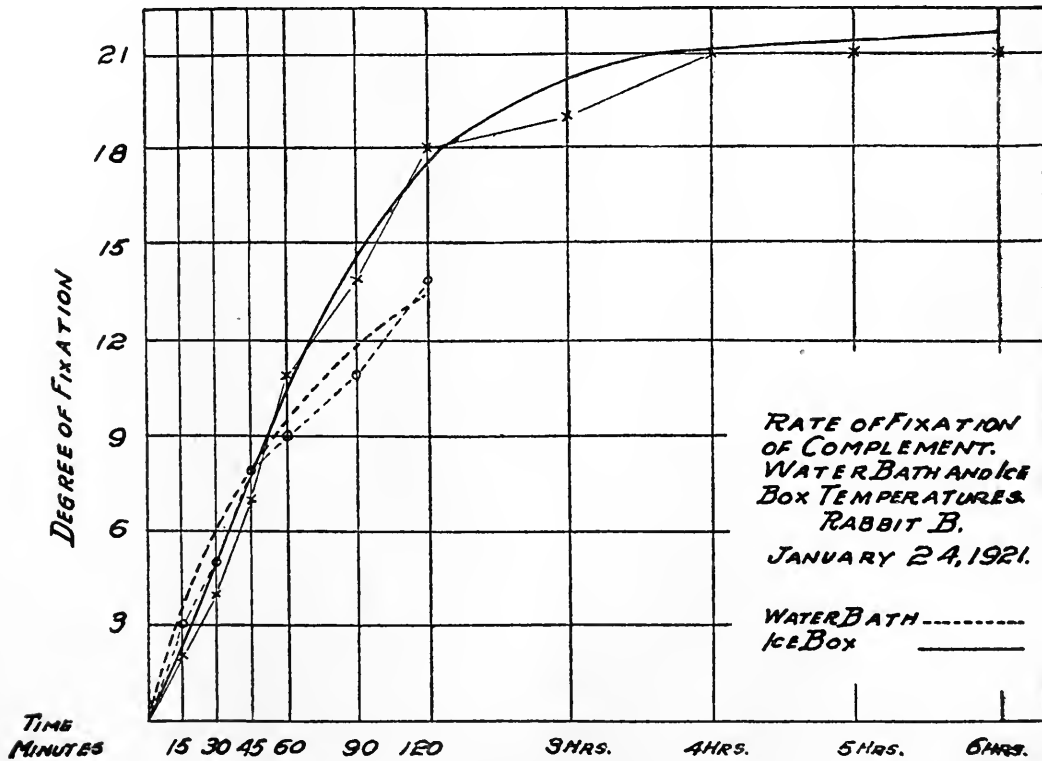
*Rate of Increase of Fixation of Complement at Water Bath and Ice Box Temperatures.*

Fixation.		Edestin immune serum, Rabbit A.									No. of positive signs, denoting degree of fixation.
Pe- riod.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
<i>min.</i>											
15	Water bath.	+	+	+	—	—	—	—	—	—	3
	Ice box.	+	+	+	—	—	—	—	—	—	3
30	Water bath.	+++	++	+	+	+	—	—	—	—	8
	Ice box.	+++	+++	++	+	+	—	—	—	—	10
45	Water bath.	++++	++++	++	+	+	—	—	—	—	11
	Ice box.	++++	++++	++	+	+	+	—	—	—	13
60	Water bath.	++++	++++	++	++	+	+	—	—	—	13
	Ice box.	++++	++++	+++	++	+	+	—	—	—	15
90	Water bath.	++++	++++	++++	+++	++	+	—	—	—	18
	Ice box.	++++	++++	++++	+++	++	+	—	—	—	18
120	Water bath.	++++	++++	++++	++++	+++	+	—	—	—	20
	Ice box.	++++	++++	++++	++++	+++	+	—	—	—	20
180	" "	++++	++++	++++	++++	+++	++	—	—	—	21
240	" "	++++	++++	++++	++++	++++	+	+	—	—	22
300	" "	++++	++++	++++	++++	++++	+	+	—	—	22

one another when the concentration of antibodies is about the same. The lesser degree of fixation at water bath temperature shown in Text-figs. 3 and 5 is difficult to explain in view of the fact that the sera of the same rabbit did not show this tendency to the same degree in Text-fig. 1.



TEXT-FIG. 1.



TEXT-FIG. 2.

Text figure 7 is given as an illustration of the nature of the curve with a weakly positive serum.

TABLE II.

*Rate of Increase of Fixation of Complement at Water Bath and Ice Box Temperatures.*

Fixation.		Phaseolin immune serum, Rabbit B.									No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
<i>min.</i>											
15	Water bath.	++	+	—	—	—	—	—	—	—	3
	Ice box.	+	+	—	—	—	—	—	—	—	2
30	Water bath.	+++	+	+	—	—	—	—	—	—	5
	Ice box.	++	+	+	—	—	—	—	—	—	4
45	Water bath.	++++	++	+	+	—	—	—	—	—	8
	Ice box.	+++	++	+	+	—	—	—	—	—	7
60	Water bath.	++++	++++	+	+	—	—	—	—	—	9
	Ice box.	++++	++++	++	+	+	—	—	—	—	11
90	Water bath.	++++	++++	++	+	—	—	—	—	—	11
	Ice box.	++++	++++	+++	++	+	—	—	—	—	14
120	Water bath.	++++	++++	+++	++	+	—	—	—	—	14
	Ice box.	++++	++++	++++	+++	++	+	—	—	—	18
180	“ “	++++	++++	++++	++++	++	+	—	—	—	19
240	“ “	++++	++++	++++	++++	++++	+	—	—	—	21
300	“ “	++++	++++	++++	++++	++++	+	—	—	—	21
360	“ “	++++	++++	++++	++++	++++	+	—	—	—	21

A consideration of the experimental data presented in this paper indicates that the affinity for complement of specific protein antigen-antibody complexes is extremely marked, fixation of complement taking place equally as well at 8–12°C. as at 37.5°C. Theoretically this is suggestive of the close relation between the phenomenon of

precipitation and that of complement fixation, the former also taking place at cold temperatures.

TABLE III.

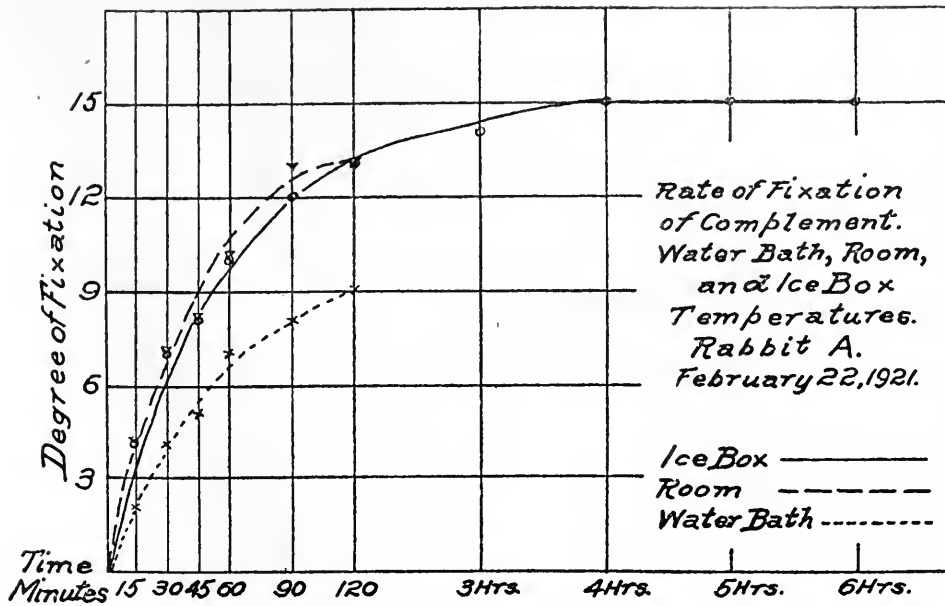
*Rate of Increase of Fixation of Complement at Water Bath, Room, and Ice Box Temperatures.*

Fixation.		Edestin immune serum, Rabbit A									No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
<i>min.</i>											
15	Water bath.	+	+	—	—	—	—	—	—	—	2
	Room.	++	+	+	—	—	—	—	—	—	4
	Ice box.	++	+	+	—	—	—	—	—	—	4
30	Water bath.	++	+	+	—	—	—	—	—	—	4
	Room.	+++	++	+	+	—	—	—	—	—	7
	Ice box.	+++	++	+	+	—	—	—	—	—	7
45	Water bath.	++	++	+	—	—	—	—	—	—	5
	Room.	+++	+++	+	+	—	—	—	—	—	8
	Ice box.	+++	+++	+	+	—	—	—	—	—	8
60	Water bath.	+++	++	+	+	—	—	—	—	—	7
	Room.	++++	+++	++	+	—	—	—	—	—	10
	Ice box.	++++	+++	++	+	—	—	—	—	—	10
90	Water bath.	+++	+++	+	+	—	—	—	—	—	8
	Room.	++++	++++	+++	+	+	—	—	—	—	13
	Ice box.	++++	++++	+++	+	—	—	—	—	—	12
120	Water bath.	+++	+++	++	+	—	—	—	—	—	9
	Room.	++++	++++	+++	+	+	—	—	—	—	13
	Ice box.	++++	++++	+++	+	+	—	—	—	—	13
180	“ “	++++	++++	+++	++	+	—	—	—	—	14
240	“ “	++++	++++	+++	++	+	+	—	—	—	15
300	“ “	++++	++++	+++	++	+	+	—	—	—	15
360	“ “	++++	++++	+++	++	+	+	—	—	—	15

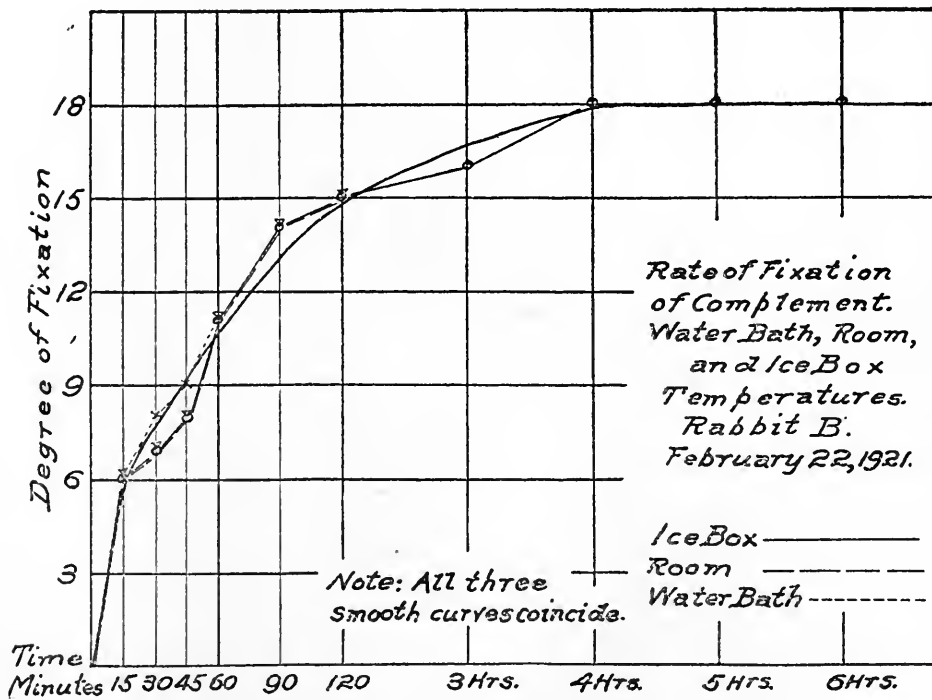
TABLE IV.

*Rate of Increase of Fixation of Complement at Water Bath, Room, and Ice Box Temperatures.*

Fixation.		Phaseolin immune serum, Rabbit B									No. of positive signs, denoting degree of fixation.
Period.	Temperature.	0.01 cc.	0.007 cc.	0.004 cc.	0.003 cc.	0.002 cc.	0.001 cc.	0.0005 cc.	0.0003 cc.	0.0001 cc.	
<i>min.</i>											
15	Water bath.	+++	++	+	—	—	—	—	—	—	6
	Room.	+++	++	+	—	—	—	—	—	—	6
	Ice box.	+++	++	+	—	—	—	—	—	—	6
30	Water bath.	+++	+++	+	+	—	—	—	—	—	8
	Room.	+++	++	+	+	—	—	—	—	—	7
	Ice box.	+++	++	+	+	—	—	—	—	—	7
45	Water bath.	++++	+++	+	+	—	—	—	—	—	9
	Room.	+++	+++	+	+	—	—	—	—	—	8
	Ice box.	+++	+++	+	+	—	—	—	—	—	8
60	Water bath.	++++	++++	++	+	—	—	—	—	—	11
	Room.	++++	++++	++	+	—	—	—	—	—	11
	Ice box.	++++	++++	++	+	—	—	—	—	—	11
90	Water bath.	++++	++++	+++	++	+	—	—	—	—	14
	Room.	++++	++++	+++	++	+	—	—	—	—	14
	Ice box.	++++	++++	+++	++	+	—	—	—	—	14
120	Water bath.	++++	++++	+++	++	+	+	—	—	—	15
	Room.	++++	++++	+++	++	+	+	—	—	—	15
	Ice box.	++++	++++	+++	++	+	+	—	—	—	15
180	“ “	++++	++++	+++	+++	+	+	—	—	—	16
240	“ “	++++	++++	++++	+++	++	+	—	—	—	18
300	“ “	++++	++++	++++	+++	++	+	—	—	—	18
360	“ “	++++	++++	++++	+++	++	+	—	—	—	18

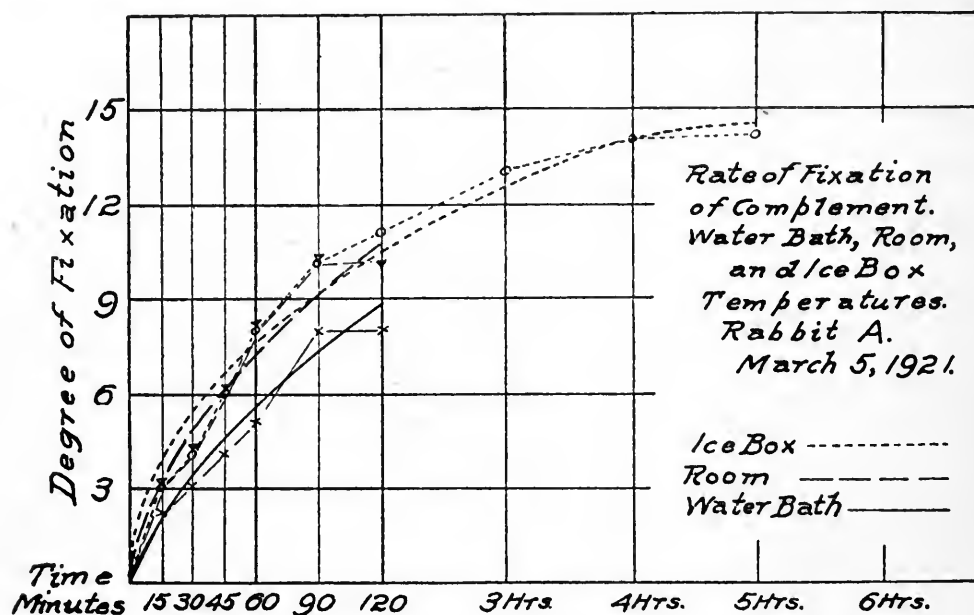


TEXT-FIG. 3.

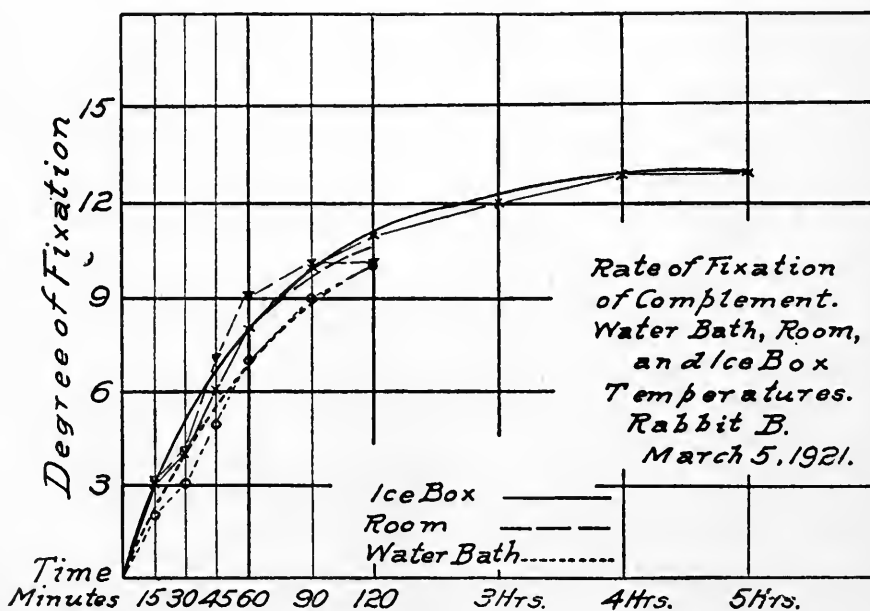


TEXT-FIG. 4.

These experiments also indicate that the rate of fixation of complement is directly proportional to the concentration of antibodies in



TEXT-FIG. 5.

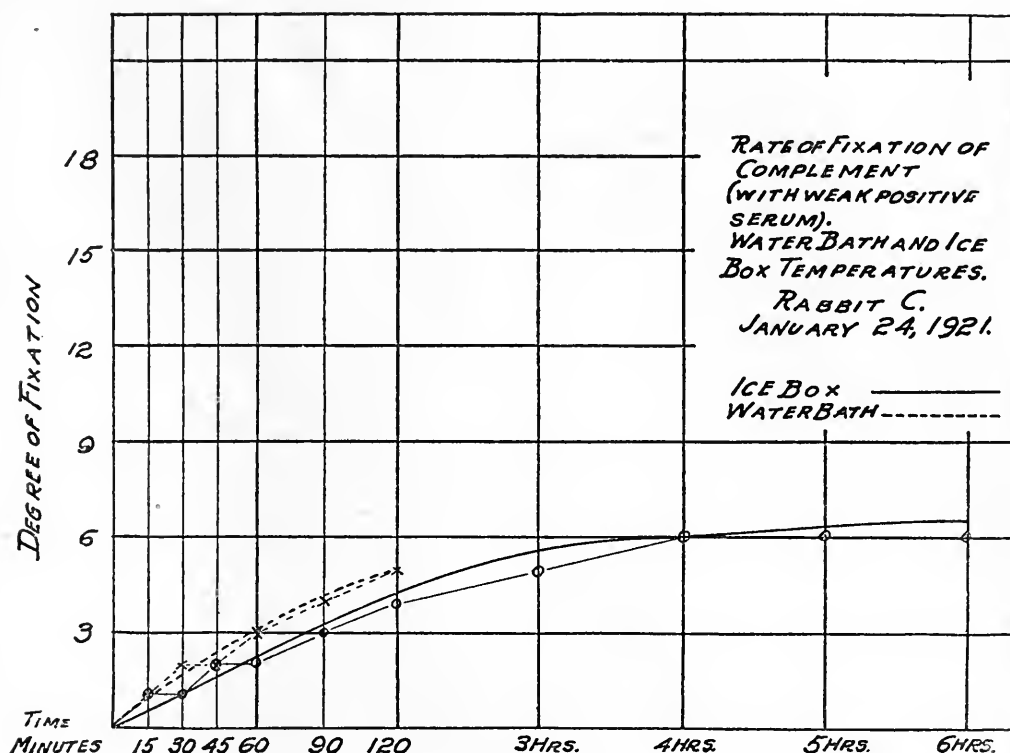


TEXT-FIG. 6.

the immune serum. This is evident by observing the various curves; the greater the concentration of antibodies, the steeper the curve.



The rate of fixation of complement at different temperatures with various Wassermann antigens and syphilitic sera will be reported in forthcoming studies.



TEXT-FIG. 7.

## CONCLUSIONS.

It is shown by complement fixation studies with protein antigens and specific immune rabbit sera that the rate of fixation of complement is determined by the concentration of antibodies in the immune sera, that the greater part of fixation of complement takes place during the 1st hour, and that fixation is practically completed at the end of 4 hours at ice box temperature.

It is further shown that the rate of fixation of complement is practically the same at ice box, room, and water bath temperatures, the tendency being for slightly stronger fixation at ice box temperature.

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# MEASUREMENT OF THE GROWTH OF TISSUES IN VITRO.

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Two fragments of the same tissue placed in identical media do not always grow at the same rate. Therefore, an acceleration or a retardation of the rate of growth of a tissue placed in an experimental medium cannot be considered as due necessarily to the action of the medium. It may be a mere accident. In order that the differences in the rate of growth of tissues, placed in media of different composition, may be attributed to an action of the medium, it must be ascertained whether these tissues would grow at the same rate, if placed in identical media. In the course of many experiments, it was attempted to find the conditions under which two parts of the same fragment of tissue cultivated in identical media would increase with the same velocity, and to develop a technique by which the increment could be accurately measured.

## *I. Technique for Obtaining the Growth of Two Fragments of Tissue at the Same Rate.*

It is already known that cultures of tissues, made under certain conditions, generally grow at about the same rate.<sup>1</sup> But in several instances, it was found that the differences in area observed after 48 hours reached 20 or even 50 per cent. The causes of these variations were carefully investigated and the reason for the inequality of the growth could always be discovered. Progressively, a number of minute details of the technique were modified and improved, and a method which renders the growth of two fragments of tissue more nearly equal was developed.

<sup>1</sup> Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 534-535.

1. *Selection of the Tissue.*—Connective tissue cells, the growth of which was the subject of the experiments, were obtained from embryonic tissues and from an old strain of fibroblasts. The growth of embryonic tissues is not regular. Moreover, it is almost impossible to cut such fragments in strictly equal parts. However, the heart of chick embryo was found to grow more regularly than other tissues. After 48 hours, a dense ring of fibroblasts has formed around the original fragment and beyond the ring are scattered fusiform or multipolar cells. Sometimes a more or less irregular layer of endothelial cells spreads into the plasma. If the presence of the peripheral cells is disregarded, and the dense ring of fibroblasts is alone taken into consideration, the area of new growth can be measured with some accuracy. The width of this ring of densely packed cells does not vary markedly, and the uniformity of its thickness can easily be ascertained. The growth consists not only of the migration and the multiplication of the cells in the medium, but also of the increase in mass of the original fragment. The increase in area can easily be measured. But, as the tissue is completely opaque, no appreciation of its thickness can be reached. However, if the fragments selected are identical, not only in surface but also in thickness, they grow at about the same rate. This condition may be obtained by the following technique: A heart taken from a chick embryo about 12 days old is opened and washed in Ringer solution. Afterwards, the walls of the ventricles are placed on a glass plate in a little Ringer solution, and square fragments are cut with a sharp cataract knife. When such a fragment is bisected, both parts are of the same thickness, and if their surface is about equal, they grow at the same rate. Often the limits of the new growth and of the primitive fragment become indistinct, and this prevents accurate appreciation of the width of the growth. Although the growth of embryonic heart is more regular than that of other organs, it is still unfitted for accurate measurement. Whenever possible, a strain of connective tissue adapted to life *in vitro* should be employed.

The tissues are taken from stock cultures of a strain of fibroblasts kept for 9 years in plasma and embryonic juices. The area of the fragment must be about 3 by 5 mm. As the tissue is not entirely opaque to light, it is easy to see whether or not its density is uniform.

Zones of greater density, where the cells are often necrotic, can be detected easily by their complete opacity to transmitted light, and they appear as white spots when they are seen by refracted light. The cultures showing these local thickenings are discarded, unless the area of greater density is symmetrical, and can be divided into two equal parts. It is very much easier to select tissues of uniform density when the old strain is used than when the cultures are made of embryonic heart. Another advantage of the old strain is that the edges of the original fragment generally remain visible for 48 hours, and that the inner and outer limits of the new tissue can be recognized. The area of the original fragment remains constant and the entire growth consists of the ring of new tissue in the medium. A third advantage of the old strain of connective tissue is that only fibroblasts grow from it. There are no ameboid cells or layers of endothelial cells, or scattered cells at the periphery of the thick ring of fibroblasts. The outline of the new growth is sharp and can be traced easily. While our experiments were made with a 9 year old strain of fibroblasts, a younger strain can be used with the same results. By placing a fragment of embryonic heart in plasma and embryonic juice, and by resecting the peripheral growth of fibroblasts when it has become very extensive, and cultivating it again, one can obtain an excellent strain of connective tissue. After ten or twelve passages, fibroblasts are practically in pure culture, grow at a regular rate, and can be used for a quantitative study of the growth of connective tissue.

2. *Preparation of the Tissue.*—The pieces of tissue used for an experiment always consisted of two or four parts of the same fragment. Fragments coming from different cultures may present differences in activity or in structural conditions which would render the rate of growth unequal. When a old strain of fibroblasts is used, the tissue is extirpated from the coagulum by four neat cuts of a sharp cataract knife, made through the peripheral growth. No part of the coagulum not yet invaded by the cells must be left around the tissue. If the incisions are not very sharp, the coagulated plasma may become folded over the cut edge, or a tractus of fibrin still adherent to the remaining coagulum may produce a dislocation of the fibrin network when the tissue is removed from the culture. When the coagulum

has been severed by the knife, the edge of the wound is slightly pushed aside with the blade, in order to make sure that no tractus of fibrin has been left adherent. After the fragment is completely detached from the surrounding coagulum, it is taken on the edge of the knife and placed in Ringer solution. It is examined again in order to make sure that the edges are not folded and that no fragment of mica is adherent to the tissue. With the cataract knife, it is divided into two parts, as equal as possible. It takes a great deal of practice to learn to divide the fragment in the proper way. If the two parts so obtained are not equal in area and in thickness, or if their edges are dilacerated, they must be discarded.

3. *Preparation of the Medium.*—The preparation of plasma, Ringer solution, and tissue juices has already been described.<sup>2</sup> It was found that still more care should be given to the possible changes of the hydrogen ion concentration of these different substances, and to the impurities which may come from the chemicals used in the salt solutions. Distilled water is redistilled in glass, and then its pH is determined. The purest chemicals are used for Ringer solution. The solutions are kept in Pyrex glassware, or in ordinary glass coated with paraffin. The pipettes are also tested in order to be sure that the alkalinity of the fluid is not increased by their contact. The H ion concentration of the serum and tissue extract may undergo progressive changes. Therefore, they should be carefully tested. Generally, while the pH of embryonic tissue juice is about 7.2 at the time of the preparation, it drops after a few days to 6.5, and if in contact with the pulp remains at that figure for a long time. The pH of plasma remains constant for several weeks. There are other changes which occur in the course of time in serum, and especially in tissue juices and extract, which make them less efficient in promoting growth and which are independent of the changes in the hydrogen ion concentration. It is, therefore, safer not to keep these substances for more than a few days in cold storage.

4. *Preparation of the Cultures.*—The fluids composing the medium are placed on the cover-glasses at the same time and thoroughly mixed. Plasma should be placed first on the surface of the glass and spread over it, and then the other substances should be added. This detail

<sup>2</sup> Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 532-533.

insures against subsequent partial or total detachment of the clot. Then the tissue fragments are taken from the Ringer solution and placed in the medium. It is important that both media should be not only of the same composition, but also of the same volume and area. The growth of a fragment of tissue in identical volumes of fluid differs when the fluid is spread at the surface of the cover-glass and when it is left in a thick drop. In order that both media may be spread equally, the cover-glass is put on a piece of paper on which a circle has been traced. After the plasma is dropped on the glass, it is spread with the point of a knife in such a way that it covers the subjacent circle. This technique allows the surface of the medium to be identical in both cultures. As cover-glasses are often of varied alkalinity, mica is substituted for glass, or both cultures are made on the same long cover-glass, in order that the conditions may be identical. Immediately after the fragments of tissue are imbedded in the medium, a hollow slide covered with a little vaseline is inverted over the cover-glasses. Care is taken that the capacity of the hollow slides is identical in both cultures. If one is larger, the evaporation of the medium is greater and its concentration increases. It is well known that a change in the concentration of the medium will modify the extent of the growth. In order to eliminate completely this cause of error, both cultures may be made on the same cover-glass and inverted over an oblong hollow slide. The cultures are then sealed at the same time, and placed in the incubator simultaneously and close together, in order that they may not be subjected to different temperatures. They are allowed to grow for 48 hours.

## *II. Measurement.*

The only means of measuring the growth of a fragment of tissue accurately would be to measure the increase of its weight. Amounts of tissue large enough to be weighed can be easily cultivated *in vitro*, but as the cells cannot be separated from the fibrin of the medium, it is impossible to use this method. The enumeration of the cells contained in the medium would allow an accurate measurement of the increase in number, but connective tissue cells grow in the shape of a tissue, and it is impossible to count them. Therefore, the increase in mass of a fragment of tissue has to be ascertained by indirect meth-

ods. The only practical way was to estimate the increase in mass by the increase in area, the thickness being kept constant. When proper care is taken, the thickness of the new tissue does not vary and can, therefore, be disregarded. The measurement of the areas of new tissue can be made by two different techniques, either by measuring the width of the ring of fibroblasts surrounding the original fragment, or by measuring the area of the new growth. The dense growth of fibroblasts which surrounds a fragment of the old strain of connective tissue after 48 hours is neatly outlined on its outer and inner border. However, when the growth is very active, the inner border, that is the edge of the primitive fragment, becomes indistinct. Generally the side which corresponds in each piece of tissue to the central part of the fragment from which it was derived can be seen distinctly, because it is thicker and darker. It sometimes happens that the growth of the side corresponding to the central part of the mother fragment grows less actively than the three peripheral sides. Only the width of the growth of symmetrical sides should be compared in both pieces. The measurements are made with an ocular micrometer. According to the activity of the tissue and the culture medium the width of the ring of new tissue varies between 0.5 and 8.00. The, old strain, when it is in active condition, produces in 48 hours a ring 7 or 8 divisions in width; that is, about 1.4 or 1.6 mm. This measurement does not take into account the increase in size of the original fragment which always occurs when embryonic heart is used. Again, it does not give precise results when, in a very active strain of connective tissue, the limit of new growth and the original fragment after 48 hours have become indistinct. In the case of the old strain, growing at a moderate rate, when the edges of the old plasma remain clear, this method has a sufficient amount of accuracy and the great advantage of being very simple and rapid.

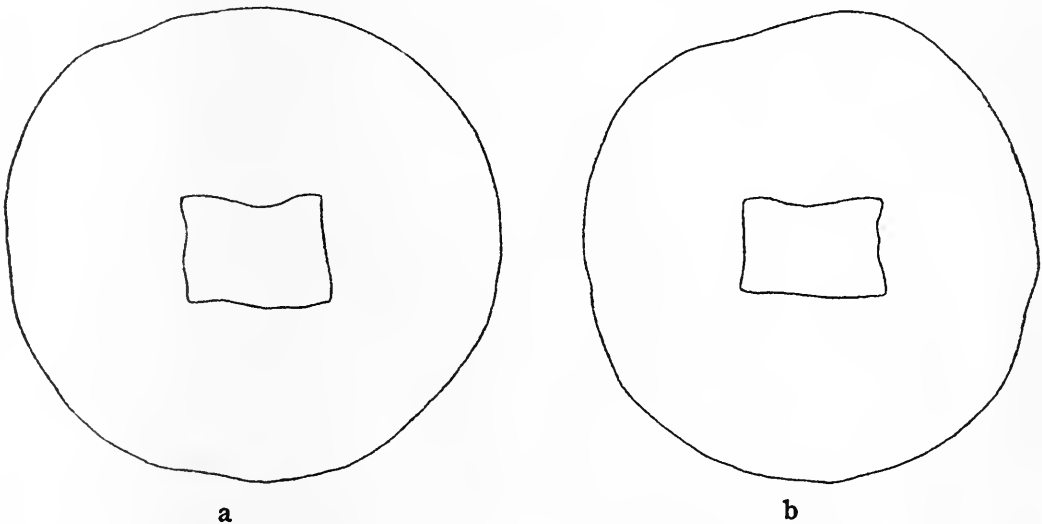
The measurement of the surface of the new growth gives more precise results, but must be made by a more elaborate technique. As soon as the cultures are completed, before the beginning of the growth, the tissues are examined. This shows whether the fragments are still the same size and thickness, whether the edges have been cut neatly, whether no fibrin remains around the tissue, whether there is no folding of the edges of the old plasma, and whether the coagula-



tion of the new medium has taken place in the proper way. If both cultures are not identical in all of these details, they should be rejected. If they are found satisfactory, they are placed in a projecting apparatus and the outline of the fragment is traced on a sheet of paper (Text-fig. 1). Care should be taken to prevent the cultures from being overheated while under the projecting apparatus, and to keep



TEXT-FIG. 1. Experiment 18539; Passage 1657. Areas of two fragments, derived from the same culture, 1 hour after transfer into fresh medium. Area a = 4.5 sq. cm. Area b = 4.1 sq. cm. Reduced about one-third off.



TEXT-FIG. 2. Experiment 18539; Passage 1657. Area of the two fragments shown in Text-fig. 1, 48 hours later. Area a = 56.25 sq. cm. Area b = 49.4 sq. cm. The relative increase of Area a is 11.5 and of Area b 11.2, and the difference is 2 per cent. Reduced about one-third off.

each one outside of the incubator about the same length of time. There is no difficulty either with fresh tissue or with the old strain in making these first drawings. The second drawing is made after 48 hours (Text-fig. 2). The original fragment is again traced and also the outline of the new growth. When the old strain is used, there is generally no difficulty in tracing the outline of the original

fragment, although the cut edges of the old plasma may be covered by so many cells that they have become indistinct. This always happens when heart tissue from a very young embryo is cultivated. The outer edge of the dense ring of fibroblasts can easily be traced. The scattered cells which may be located beyond the limits of the dense growth should be disregarded. If the thickness of the new tissue is irregular, or if the outer edge is irregular and indistinct, the culture should be rejected.

Afterwards, the area of the original fragment and the total area after 48 hours are measured with the planimeter and expressed in square centimeters. The total area minus the area of the original fragment represents the absolute increase of the fragment. This area divided by the area of the primitive fragment is the relative increase of the fragment. The relative increase of the larger fragment divided by the relative increase of the smaller fragment expresses the difference in growth.

### *III. Results Obtained with Two Fragments of the 9 Year Old Strain of Connective Tissue.*

Two series of thirty-two experiments were made, the results of which are shown in Tables I and II. Table I is composed of experiments made with a technique previously described,<sup>3</sup> in which no special care was taken in the selection of the fragments of tissues. In seventeen experiments, the differences between the growth of the two fragments are less than 10 per cent. In nine, it varies between 10 and 20 per cent; in four, between 20 and 30 per cent, and in the last two, the difference in one is 33 per cent, and in the other 51 per cent.

The results of the thirty-two experiments contained in Table II show the value of the improved technique. In twenty-five experiments, the difference in growth is less than 6 per cent; in seven, it varies between 6 and 7 per cent.

These results mean that this technique can be used profitable when the factors acting on the rate of growth are supposed to increase it by more than 10 per cent.

<sup>3</sup> Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531-534.

TABLE I.

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		sq. cm.	sq. cm.			
1	16691-1 16691-2	June 5	1565	2.3 2.5	40.7 44.1	17.7 17.64	1.0	0.0
2	16694-1 16694-2	" 5	1565	2.0 2.2	30.3 36.4	15.5 16.54	1.09	0.09
3	16695-1 16695-2	" 5	1565	4.7 5.0	66.0 77.3	14.04 15.46	1.12	0.12
4	17029-1 17029-2	July 8	1581	3.0 2.6	71.9 55.4	23.96 21.3	1.12	0.12
5	17030-1 17030-2	" 8	1581	4.2 3.4	91.5 68.8	21.78 20.23	1.08	0.08
6	17031-1 17031-2	" 8	1581	3.1 3.5	53.1 66.5	17.13 19.0	1.10	0.10
7	17032-1 17032-2	" 8	1581	3.7 4.0	97.0 80.5	26.21 20.12	1.26	0.26
8	17059-1 17059-2	" 12	1583	2.1 2.0	34.0 31.3	16.2 15.5	1.04	0.04
9	17062-1 17062-2	" 12	1583	2.1 2.1	42.6 40.9	20.2 19.47	1.04	0.04
10	17066-1 17066-2	" 12	1583	3.6 4.0	38.6 39.0	10.72 9.75	1.09	0.09
11	17075-1 17075-2	" 12	1582	1.6 2.0	39.0 30.6	24.47 15.3	1.51	0.51
12	17140-1 17140-2	" 20	1587	5.8 5.0	46.7 43.3	8.05 8.66	1.07	0.07
13	17143-1 17143-2	" 20	1588	3.2 3.4	49.1 43.2	15.34 12.7	1.20	0.20
14	17145-1 17145-2	" 20	1588	3.5 3.7	36.5 29.1	10.45 7.86	1.33	0.33
15	17146-1 17146-2	" 20	1588	2.5 2.8	34.2 44.3	13.68 15.82	1.15	0.15
16	17148-1 17148-2	" 20	1586	3.1 3.5	48.3 42.6	15.58 12.17	1.28	0.28

TABLE I—*Concluded*

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		sq. cm.	sq. cm.			
17	17200-1 17200-2	July 26	1591	4.0 3.5	49.7 55.6	12.42 14.88	1.19	0.19
18	17201-1 17201-2	" 26	1591	3.5 3.9	55.6 58.7	14.88 14.05	1.06	0.06
19	17202-1 17202-2	" 26	1589	3.1 3.5	66.3 64.7	21.38 18.48	1.15	0.15
20	17203-1 17203-2	" 26	1589	4.7 5.2	63.8 72.1	13.57 13.86	1.02	0.02
21	17207-1 17207-2	" 26	1589	5.5 6.3	75.3 72.7	13.67 11.54	1.19	0.19
22	17580-1 17580-2	Sept. 13	1613	6.6 4.5	92.2 78.8	13.9 17.4	1.25	0.25
23	17586-1 17586-2	" 13	1613	5.4 5.4	67.9 64.2	12.5 11.9	1.05	0.05
24	17589-1 17589-2	" 13	1613	4.7 5.2	78.9 76.7	16.7 14.7	1.13	0.13
25	17591-1 17591-2	" 13	1613	4.5 5.3	42.1 50.4	9.3 9.5	1.02	0.02
26	17592-1 17592-2	" 13	1613	5.5 5.8	81.8 83.6	14.8 14.4	1.02	0.02
27	18120-1 18120-2	Nov. 8	1643	2.5 2.6	42.5 40.8	17.0 15.7	1.08	0.08
28	18121-1 18121-2	" 8	1643	4.5 4.6	52.0 51.9	11.6 11.3	1.01	0.01
29	18122-1 18122-2	" 8	1643	3.0 3.2	43.0 44.2	16.3 13.8	1.18	0.18
30	18282-1 18282-2	" 20	1648	4.9 5.0	59.1 62.6	12.1 12.5	1.03	0.03
31	18285-1 18285-2	" 20	1468	6.9 6.1	52.1 49.9	7.55 8.3	1.09	0.09
32	18289-1 18289-2	" 20	1648	6.4 7.0	64.6 70.0	10.1 10.0	1.0	0.09

TABLE II.

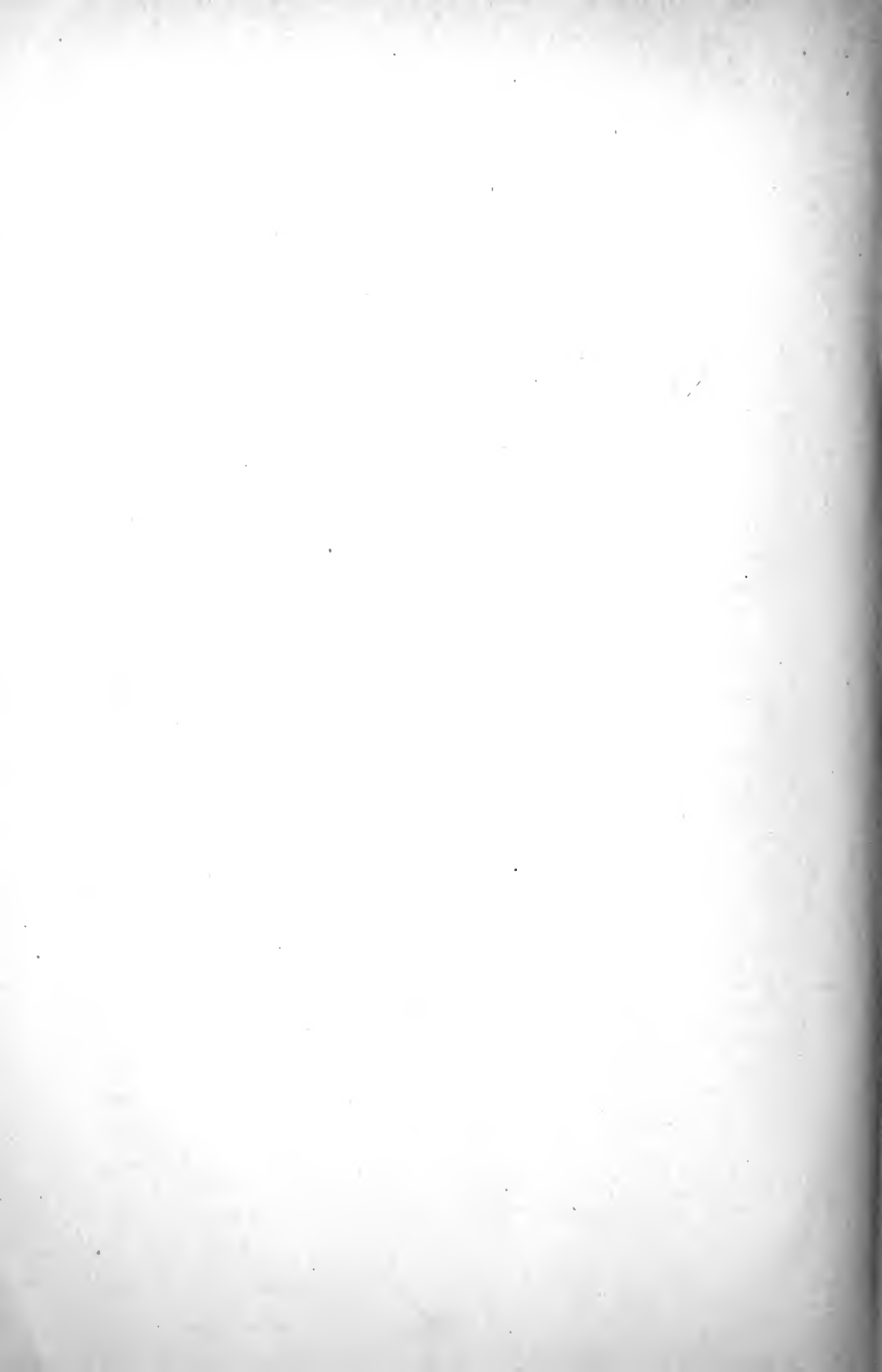
No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		sq. cm.	sq. cm.			
1	18334-1 18334-2	Nov. 24	1650	4.2 3.0	50.3 38.3	12.0 12.73	1.06	0.06
2	18337-1 18337-2	" 24	1648	4.4 4.3	56.2 55.2	12.8 12.9	1.0	0.0
3	18340-1 18340-2	" 24	1650	6.2 5.4	47.6 44.9	7.7 8.3	1.07	0.07
4	18416-1 18416-2	" 29	1653	3.5 3.7	49.9 48.6	14.2 13.5	1.05	0.05
5	18417-1 18417-2	" 29	1653	4.0 4.0	66.9 68.2	15.7 16.0	1.05	0.05
6	18419-1 18419-2	" 29	1653	2.0 2.2	30.3 36.4	15.5 16.5	1.06	0.06
7	18449-1 18449-2	Dec. 1	1654	3.0 3.2	60.0 69.9	20.0 20.84	1.04	0.04
8	18462-1 18462-2	" 1	1654	2.8 3.0	30.24 33.1	10.8 11.0	1.02	0.02
9	18463-1 18463-2	" 1	1654	3.3 3.9	50.2 61.6	15.5 15.8	1.02	0.02
10	18516-1 18516-2	" 4	1654	3.9 2.9	49.7 37.7	12.74 13.0	1.02	0.02
11	18518-1 18518-2	" 4	1655	3.7 3.9	43.3 44.8	11.7 11.43	1.05	0.05
12	18522-1 18522-2	" 4	1656	4.1 4.1	53.2 57.3	12.97 13.97	1.07	0.07
13	18534-1 18534-2	" 6	1657	1.8 1.7	34.6 33.3	19.2 19.6	1.02	0.02
14	18535-1 18535-2	" 6	1657	3.7 3.8	39.3 42.1	10.5 10.01	1.03	0.03
15	18539-1 18539-2	" 6	1657	4.5 4.1	51.75 45.3	11.5 11.2	1.02	0.02
16	18575-1 18575-2	" 8	1658	2.3 2.5	40.7 44.1	17.7 17.64	1.0	0.0

TABLE II—*Concluded.*

No. of experiment.	Culture No.	Date.	Passage No.; old strain.	Surface area of fragment.	Surface area of growth.	Relative increase.	Ratio.	Difference.
		1920		sq. cm.	sq. cm.			
17	18579-1 18579-2	Dec. 8	1658	5.0 5.0	60.1 61.2	12.0 12.2	1.01	0.01
18	18580-1 18580-2	" 8	1658	4.3 4.2	50.7 49.2	11.66 11.66	1.0	0.0
19	18621-1 18621-2	" 11	1659	5.2 5.3	63.6 65.5	12.21 12.17	1.02	0.02
20	18625-1 18625-2	" 11	1660	3.5 4.2	73.0 77.1	20.85 21.0	1.01	0.01
21	18628-1 18628-2	" 11	1660	4.7 5.0	64.0 71.5	14.01 14.3	1.01	0.01
22	18768-1 18768-2	" 20	1664	5.1 5.0	67.8 70.1	13.3 14.02	1.06	0.06
23	18773-1 18773-2	" 20	1665	4.7 4.9	68.3 67.7	14.47 13.69	1.05	0.05
24	18777-1 18777-2	" 20	1667	5.1 5.0	77.6 86.4	16.1 17.28	1.07	0.07
25	18904-1 18904-2	" 26	1668	2.5 2.5	42.8 40.8	17.12 16.32	1.05	0.05
26	18908-1 18908-2	" 26	1667	4.6 4.7	76.8 73.4	16.68 16.68	1.0	0.0
27	18909-1 18909-2	" 26	1667	3.7 5.0	68.8 88.1	18.52 17.62	1.04	0.04
		1921						
28	19015-1 19015-2	Jan. 1	1669	4.4 4.1	25.0 23.8	5.68 5.8	1.02	0.02
29	19024-1 19024-2	" 1	1670	3.3 3.2	62.2 62.7	18.88 19.6	1.03	0.03
30	19309-1 19309-2	" 24	1682	3.5 4.6	16.06 20.5	3.59 3.47	1.03	0.03
31	19320-1 19320-2	" 25	1682	3.7 4.6	30.7 36.5	8.56 7.96	1.07	0.07
32	19495-1 19495-2	Feb. 7	1683	3.7 4.4	32.1 38.1	8.67 8.65	1.0	0.0

## CONCLUSIONS.

1. A technique has been developed by which two fragments of tissue, put in identical media, can grow at almost the same rate.
2. A method of measuring the growing tissue is described.
3. The differences observed in the rate of growth of two fragments of the 9 year old strain of connective tissue, cultivated in identical media and measured according to this technique, are less than 10 per cent.





## STUDIES ON PNEUMOCOCCUS IMMUNITY.

### I. ACTIVE IMMUNIZATION OF MONKEYS AGAINST PNEUMOCOCCUS TYPE I PNEUMONIA WITH PNEUMOCOCCUS TYPE I VACCINE.\*

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In an article recently published on the results of prophylactic vaccination against pneumococcus pneumonia in monkeys, Cecil and Blake<sup>1</sup> found that subcutaneous inoculation with Pneumococcus Type I vaccine in doses comparable to those employed in man did not protect monkeys against subsequent attacks of Pneumococcus Type I pneumonia, either spontaneous or experimental. Vaccination did, however, modify the course of the disease. Invasion of the blood stream by the pneumococcus was usually slight or absent in vaccinated animals, and the proportion of recoveries was considerably higher for vaccinated than for unvaccinated monkeys. In the experiments of Cecil and Blake, comparatively small doses of vaccine were used. In view of the failure, however, of small doses of vaccine to give complete protection against pneumonia, it seemed desirable to determine the effect of large doses of pneumococcus vaccine.

In the following experiments, three species of monkeys have been employed. *Macacus rhesus* was used in three of the experiments, *Macacus syrichtus* in one, and the *Cebus capucinus* in one. *Macacus rhesus* is less susceptible to pneumococcus infection than the other two species, and rarely if ever develops a true lobar pneumonia. The infection is more apt to be of the interstitial or confluent lobular type. The Philippine macaque (*Macacus syrichtus*) is the preferable animal for studying experimental pneumococcus pneumonia, as it develops a

\* Part of the funds for carrying on this work were provided by the Influenza Commission of the Metropolitan Life Insurance Company, New York.

<sup>1</sup> Cecil, R. L., and Blake, F. G., *J. Exp. Med.*, 1920, xxxi, 519.

true lobar consolidation. At the time these experiments were carried out, however, it was not possible to obtain this species in any considerable number. *Cebus capucinus* occupies an intermediate position in respect to susceptibility to pneumococcus pneumonia. In some cases it may present a typical lobar consolidation, but, more often, this species, like the *rhesus*, develops only an interstitial or patchy consolidation, which is usually associated with a heavy pneumococcus septicemia.

### *Methods.*

The vaccine employed for these experiments was prepared from a stock laboratory strain of *Pneumococcus* Type I. This organism was highly virulent, killing a mouse in quantities as small as 0.0000001 cc. of a 24 hour broth culture. The vaccine was prepared as follows: 18 to 24 hour glucose broth cultures were centrifuged, the sediment was washed with normal salt solution, and then resuspended in normal salt solution in such a concentration that 0.5 cc. equalled 20 billion pneumococci. The suspension was heated to 55° C. for 1 hour to kill the pneumococci, and 0.25 per cent tricresol added as a preservative. With one exception (Experiment 3), the same dosage of vaccine was employed in all the experiments. Three subcutaneous injections were given at intervals of 1 week, the first dose consisting of 20 billion pneumococci, the second of 40 billion, and the third of 60 billion. The injections were made in the abdominal wall, and caused very little local or general reaction.

The culture used for testing the immunity of the vaccinated monkeys was the same strain of *Pneumococcus* Type I from which the vaccine had been prepared. The method of producing experimental pneumonia in monkeys has been previously described by Blake and Cecil.<sup>2</sup> A small quantity of an 18-hour broth culture of pneumococcus is introduced with a Luer syringe through the skin into the trachea. In testing for resistance to pneumonia following the injection of pneumococcus vaccine, the intratracheal inoculations were made in most cases 2 to 3 weeks after the completion of vaccination. The dose of culture injected varied from 0.01 to 0.000001 cc. for each monkey. A further test of the immunity of each vaccinated monkey was made

<sup>2</sup> Blake, F. G., and Cecil, R. L., *J. Exp. Med.*, 1920, xxxi, 403.

by withdrawing some blood just before intratracheal inoculation, and carrying out protection tests in mice with the serum of the monkey.

In most cases the monkeys died or were killed at the conclusion of the experiment. Complete autopsies were performed in each instance and microscopic sections studied from the various lobes of the lungs.

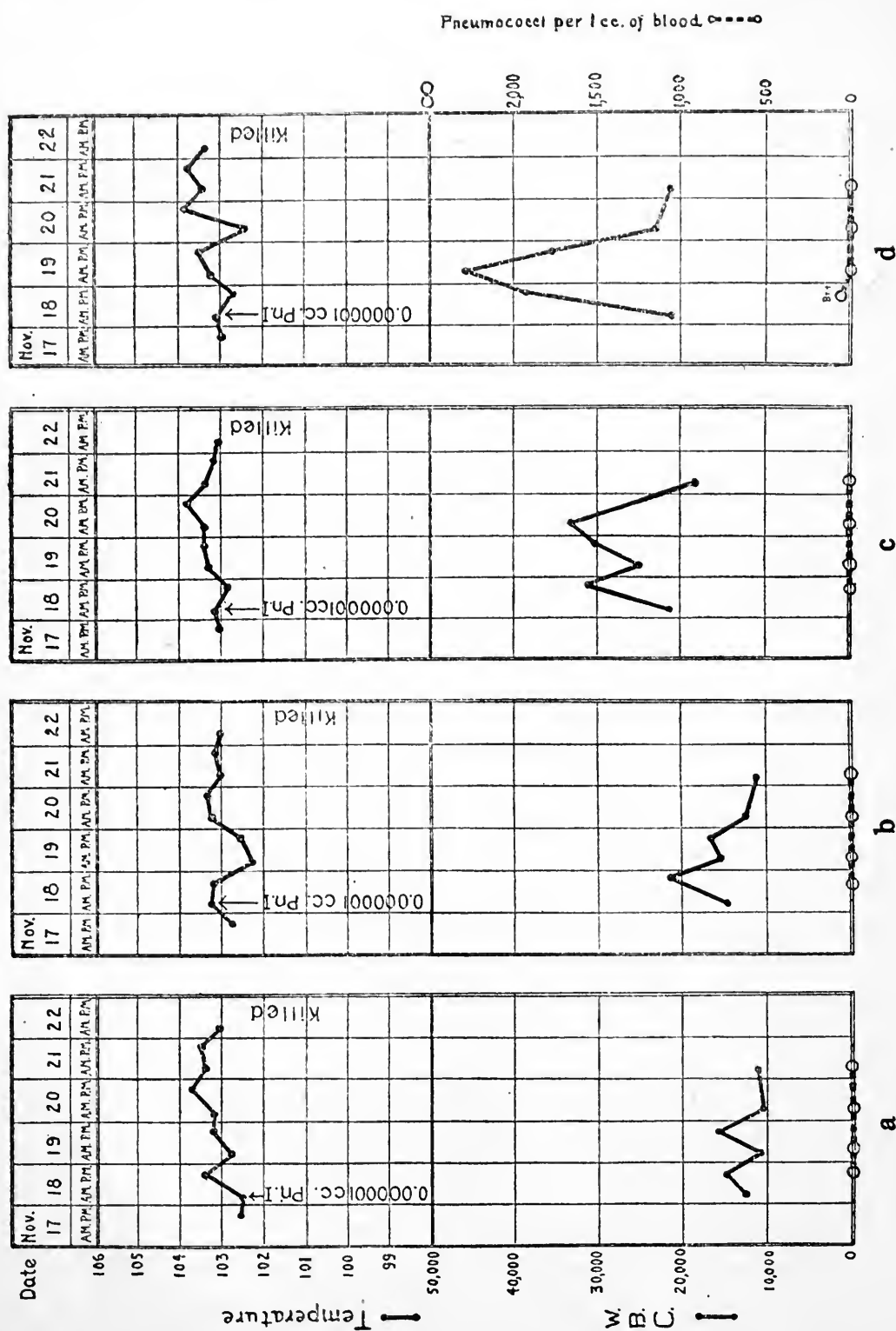
*Active Immunity in Macacus rhesus Following Vaccination with Large Doses of Pneumococcus Type I Vaccine.*

The first experiment with large doses of *Pneumococcus* Type I vaccine was carried out on *rhesus* monkeys.

*Experiment 1.*—Oct. 21, 1919. Three *Macacus rhesus* monkeys (Nos. 1 to 3) received each 20 billion (0.5 cc.) *Pneumococcus* Type I saline vaccine subcutaneously. Oct. 22. Mild local reactions. Oct. 28. Each monkey received 40 billion (1 cc.) *Pneumococcus* Type I vaccine subcutaneously. Nov. 4. Each monkey received 60 billion (1.5 cc.) *Pneumococcus* Type I vaccine subcutaneously. Nov. 18. 2 weeks after last injection of vaccine, each of the three monkeys received 0.000001 cc. of an 18hour broth culture of *Pneumococcus* Type I intratracheally. An unvaccinated control monkey (No. 12) received the same amount of culture intratracheally.

The results are shown in Text-fig. 1. The three vaccinated monkeys remained perfectly well, showed practically no change in temperature or leucocyte count, and had negative blood cultures throughout the period of observation. The control was sick for 3 days, developed a leucocytosis of 47,000, and had a transient pneumococcus septicemia. All four animals were killed on the 5th day following inoculation. The lungs in the vaccinated monkeys were entirely free from pneumonia. In the control a small patch of interstitial pneumonia was found in the left lower lobe. Cultures from the lungs and heart's blood were sterile in all the monkeys.

*Protection Tests.*—The serums from the three vaccinated monkeys were tested for specific protective bodies against *Pneumococcus* Type I. On November 18, just before intratracheal inoculation, the three vaccinated monkeys were bled for protection tests in mice. The serum of Monkey 1 developed a moderate amount of protective substance, protecting a mouse against 0.0000001 cc. of *Pneumococcus* Type I culture. The serum of Monkey 2 protected mice against 0.0001 cc. of *Pneumococcus* Type I culture. The serum of Monkey 3 showed practically no protective power.



TEXT-FIG. 1, *a* to *d*. Active immunity against Pneumococcus Type I pneumonia following vaccination with Pneumococcus Type I vaccine. (*a*), (*b*), and (*c*) Monkeys 1, 2, and 3; each received 120 billion Pneumococcus Type I, vaccine subcutaneously. (*d*) Monkey 12; control. Br.+ , Broth culture positive for Pneumococcus Type I.

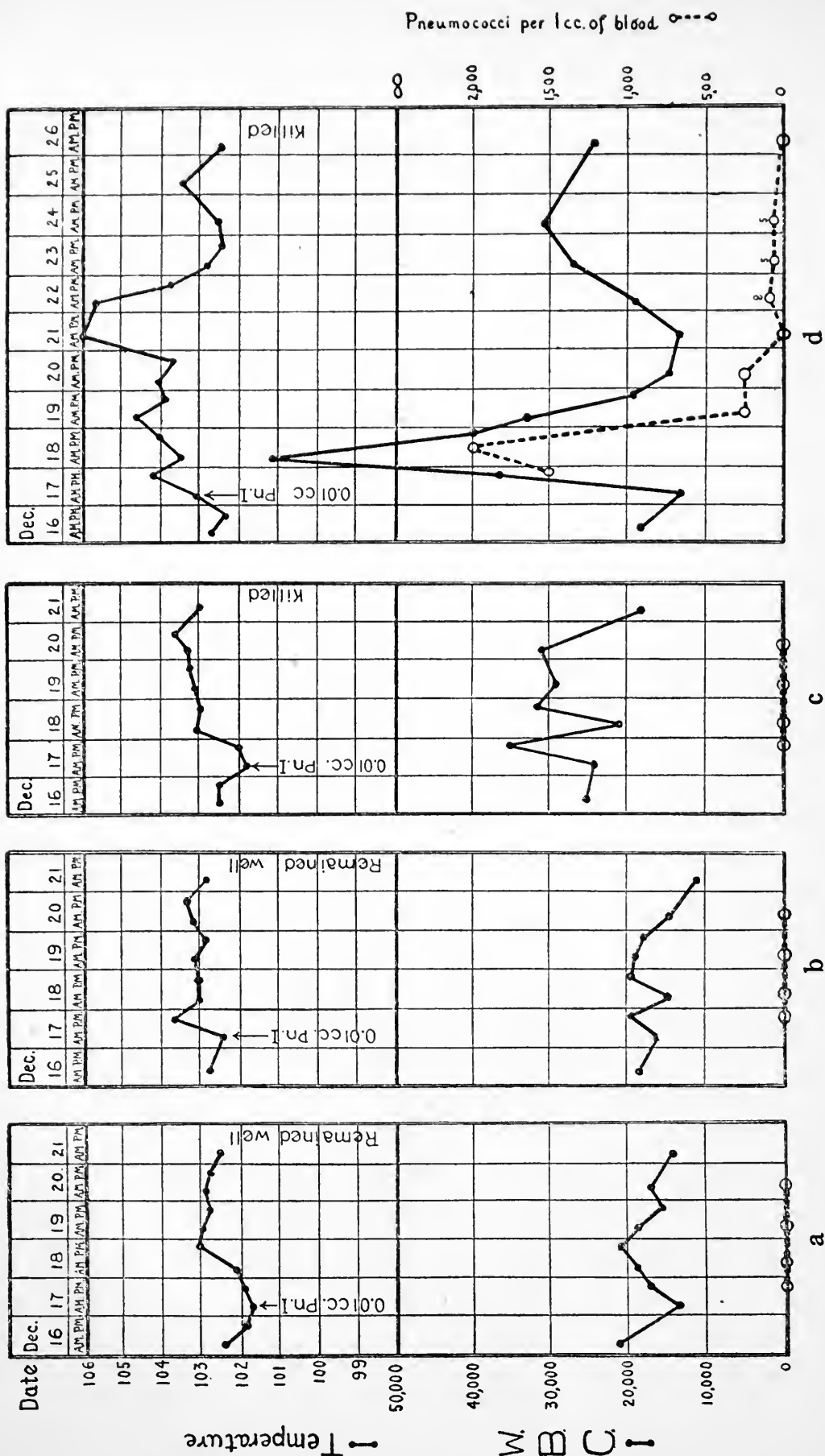
It is evident from this experiment that three large subcutaneous doses of *Pneumococcus* Type I vaccine conferred complete protection against pneumonia. The result, however, was unsatisfactory in one respect; the control developed only a small patch of interstitial pneumonia and was not seriously ill. The experiment was therefore repeated on the same species with larger infecting doses of *Pneumococcus* Type I culture.

*Experiment 2.*—Nov. 24, 1919. Three *Macacus rhesus* monkeys (Nos. 13 to 15) received each 20 billion (0.5 cc.) *Pneumococcus* Type I saline vaccine subcutaneously. Nov. 27. Small nodules at site of vaccine injection. Dec. 1. Each monkey received 40 billion (1 cc.) *Pneumococcus* Type I vaccine subcutaneously. Dec. 6. Small nodules at site of previous inoculation. In Monkey 13 the nodule has broken down and is discharging sterile pus. Dec. 7. Each monkey received 60 billion (1.5 cc.) *Pneumococcus* Type I vaccine subcutaneously. Dec. 17. Each monkey received 0.01 cc. of an 18 hour broth culture of *Pneumococcus* Type I intratracheally, and an unvaccinated control monkey (No. 21) received the same amount (0.01 cc.) of culture intratracheally.

The results are shown in Text-fig. 2. The three vaccinated monkeys remained lively and well, and showed no noteworthy changes in temperature or leucocyte count. The blood remained sterile in the three vaccinated monkeys. The control monkey developed the signs and symptoms of pneumonia and recovered by crisis on the 6th day. One of the vaccinated monkeys (No. 15) and the control monkey were killed. The vaccinated monkey showed normal lungs. The control showed a resolving pneumonia of the interstitial type involving the right upper, middle, and lower lobes and the left upper lobe. Cultures taken at autopsy from the lungs and heart's blood of both monkeys were sterile.

*Protection Tests.*—Blood was taken from the veins of the three vaccinated monkeys just previous to the intratracheal inoculations for the purpose of determining the protective power of their serums. In spite of the high degree of active immunity conferred by the vaccine, none of the serums showed any protection for mice.

This experiment corroborated the findings in Experiment 1 and indicates that three large subcutaneous injections of *Pneumococcus* Type I vaccine confer complete protection against the homologous type of pneumonia.



TEXT-FIG. 2, *a* to *d*. Active immunity against Pneumococcus Type I-pneumonia following vaccination with Pneumococcus Type I vaccine. (*a*), (*b*), and (*c*) Monkeys 13, 14, and 15; each received 120 billion Pneumococcus Type I vaccine subcutaneously. (*d*) Monkey 21; control.

It should be noted that in these experiments, as in the previously reported experiments of Cecil and Blake, the amount of protective substance in the serum of vaccinated monkeys varies widely with different individuals and apparently bears no close relation to local immunity in the lungs, a phenomenon which appears to be fairly constant following vaccination.

*Active Immunity Following Intravenous Vaccination with Small Doses of Pneumococcus Type I Vaccine.*

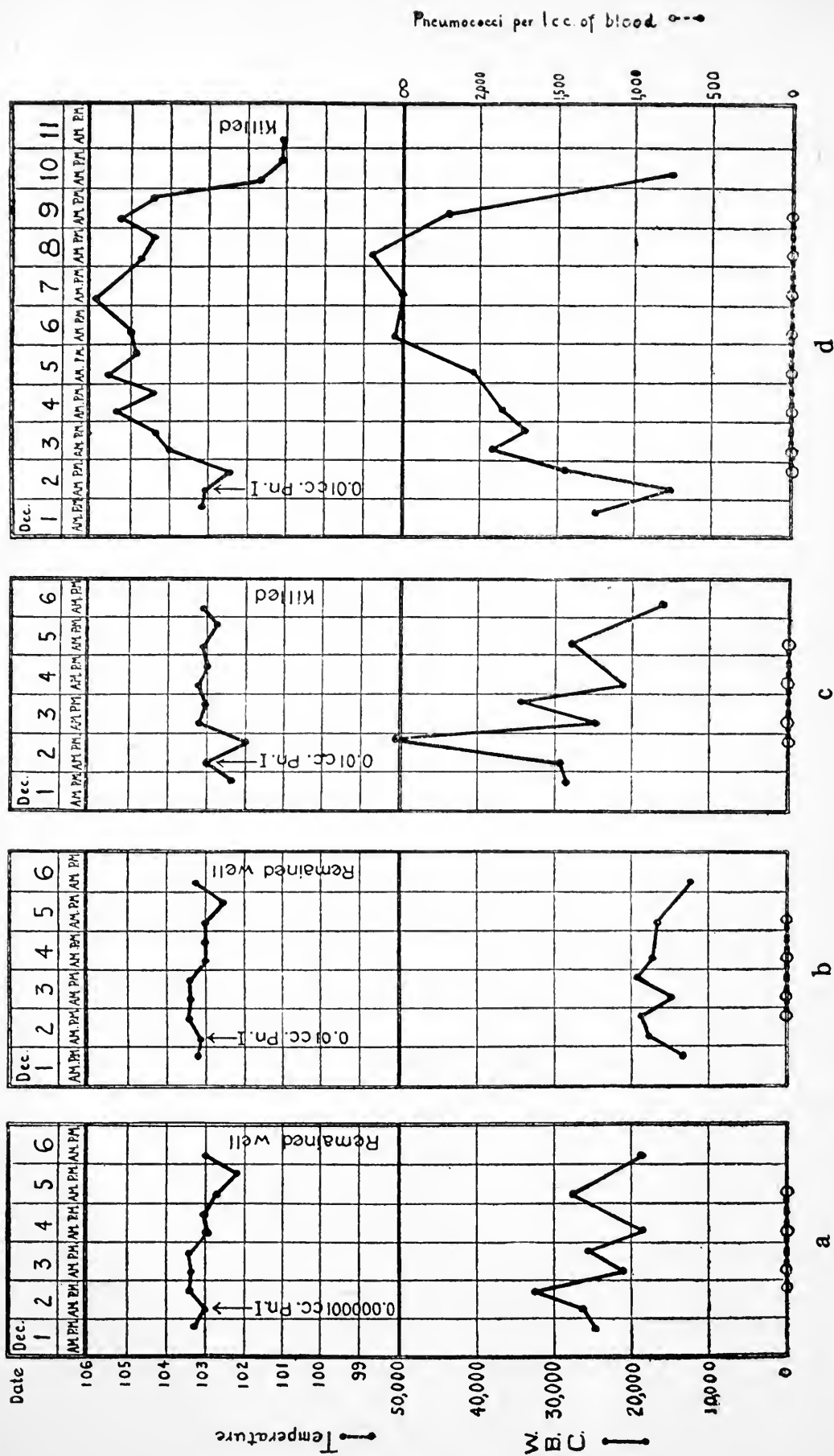
In order to determine the effect of intravenous injections of pneumococcus vaccine, three *rhesus* monkeys were injected intravenously with small doses of *Pneumococcus* Type I vaccine and their immunity was tested by intratracheal inoculation of virulent pneumococci as in the preceding experiments.

*Experiment 3.*—Oct. 27, 1919. Three *Macacus rhesus* monkeys (Nos. 4, 5, and 10) received each 300 million *Pneumococcus* Type I saline vaccine intravenously. Nov. 3. Each monkey received 400 million *Pneumococcus* Type I vaccine intravenously. Nov. 4. Monkeys lively and well. Nov. 10. Each monkey received 800 million *Pneumococcus* Type I vaccine intravenously. Nov. 11. Animals lively and well. Nov. 17. Each monkey received 1,600 million *Pneumococcus* Type I vaccine intravenously. Dec. 2. Each monkey received 0.01 cc. of an 18 hour broth culture of a *Pneumococcus* Type I intratracheally. An unvaccinated control monkey (No. 17) received the same amount (0.01 cc.) of culture intratracheally.

The results are shown in Text-fig. 3. The three vaccinated monkeys remained lively and well. Monkey 10 had a sharp leucocyte reaction but showed no other signs of infection. The control monkey became ill and showed symptoms of pneumonia. One of the vaccinated monkeys (No. 10) and the control monkey (No. 17) were killed. Monkey 10 showed an acute bronchitis but no pneumonia. The control monkey presented a complete consolidation of the right upper lobe, and, microscopically, resolving pneumonia of the interstitial type was demonstrated. Cultures taken at autopsy from the lungs and heart's blood of these two monkeys were in both cases sterile.

*Protection Tests.*—The vaccinated monkeys were bled previous to the intratracheal inoculations in order to test the protective power of their serums. Monkey 10 showed a high degree of protective





TEXT-FIG. 3, a to d. Active immunity against Pneumococcus Type I pneumonia following vaccination with Pneumococcus Type I vaccine. (a), (b), and (c) Monkeys 4, 5, and 10; each received 3 billion Pneumococcus Type I vaccine intravenously. (d) Monkey 17; control.



substance in its serum which protected mice against 0.0001 cc. of *Pneumococcus* Type I culture; in Monkey 4 there was a small amount of protective substance, the mouse receiving 0.0000001 cc. of *Pneumococcus* Type I surviving. The serum of Monkey 5 showed no protective power at all.

This experiment demonstrates that small doses of *Pneumococcus* Type I vaccine injected intravenously confer an immunity equivalent to that obtained by large doses of pneumococcus vaccine administered subcutaneously. This is in harmony with the observations of many immunologists that immunity can be obtained more readily by intravenous than by subcutaneous inoculations.

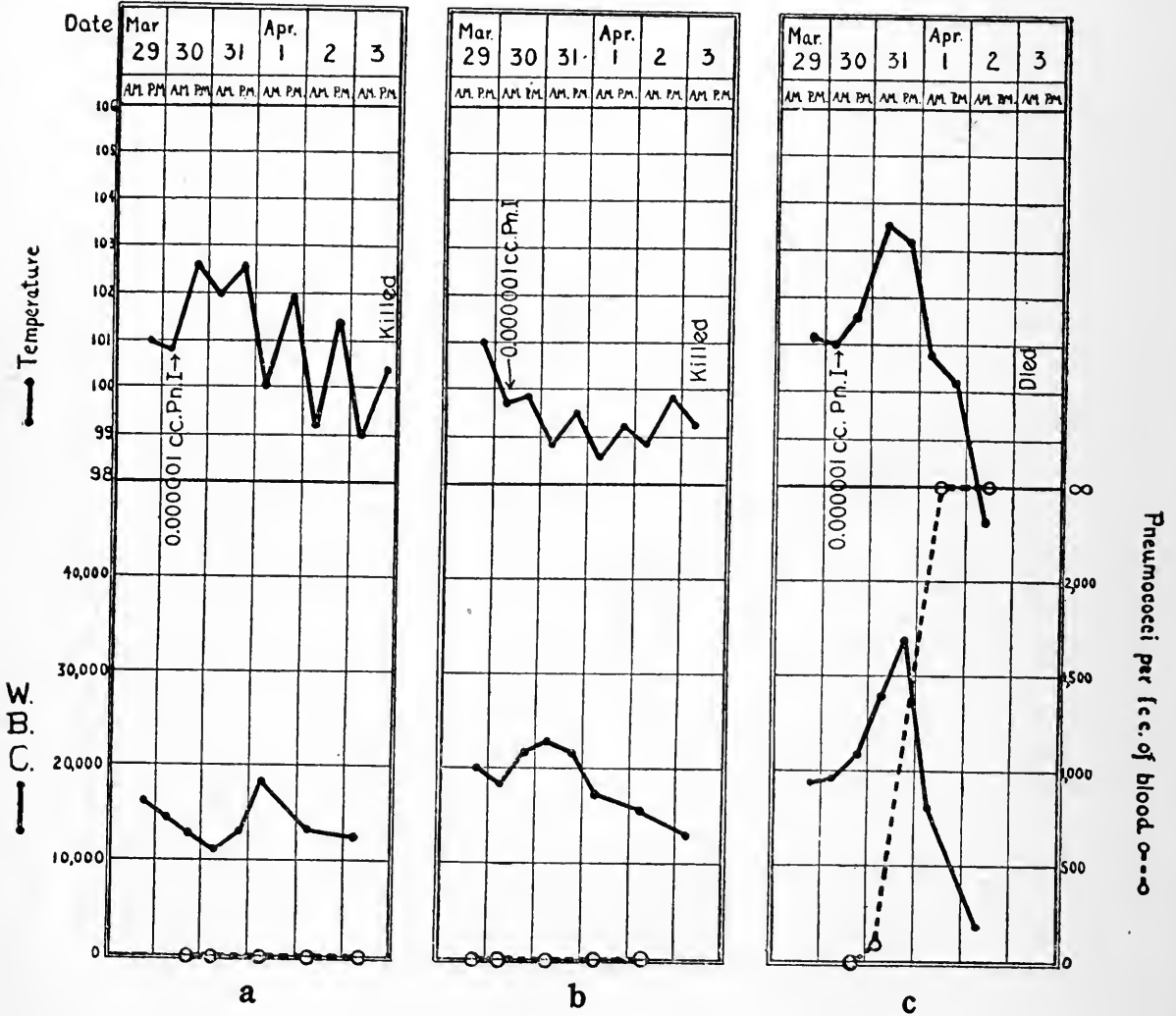
*Active Immunity in Philippine Monkeys Following Vaccination with Large Doses of Pneumococcus Type I Vaccine.*

In view of the fact that most of the experiments carried out by Cecil and Blake in their study of prophylactic vaccination against pneumonia in monkeys were performed on *Macacus syrichtus*, it seemed desirable to control the result obtained in the above described experiments by an experiment on Philippine monkeys.

*Experiment 4.*—Feb. 27, 1920. Two Philippine monkeys (Nos. 34 and 35) received each 20 billion (0.5 cc.) *Pneumococcus* Type I saline vaccine subcutaneously. Feb. 28. Monkeys lively and well. Mar. 5. Each monkey received 40 billion (1 cc.) *Pneumococcus* Type I vaccine subcutaneously. Mar. 12. Each monkey received 60 billion (1.5 cc.) *Pneumococcus* Type I vaccine subcutaneously. Mar. 30. Each monkey received 0.000001 cc. of an 18 hour broth culture of *Pneumococcus* Type I intratracheally. An unvaccinated control monkey (No. 36) received the same amount (0.000001 cc.) of culture intratracheally.

The results are shown in Text-fig. 4. The two vaccinated monkeys remained lively and well. Their blood remained free from bacteria and the temperature and leucocytes showed no significant variation. The control monkey developed pneumonia and an overwhelming pneumococcus septicemia and died on the 5th day of the disease. The two vaccinated monkeys were killed on the 5th day following inoculation. Monkey 34 showed several clusters of small gray tubercles but there was no pneumonic consolidation. The presence of tuberculosis in this monkey explains the morning and evening varia-

tions in temperature (Text-fig. 4). Monkey 35 also showed a patch of encapsulated tuberculosis in the left upper lobe but there was no fresh consolidation. The control monkey showed an early pneumococcus pneumonia (stage of engorgement), involving the right and



TEXT-FIG. 4, *a* to *c*. Active immunity against *Pneumococcus* Type I pneumonia in Philippine monkeys following vaccination with *Pneumococcus* Type I vaccine. (*a*) and (*b*) Monkeys 34 and 35; each received 120 billion *Pneumococcus* Type I vaccine subcutaneously. (*c*) Monkey 36; control.

left lower lobes. Cultures at autopsy from the lungs and heart's blood of the vaccinated monkey were sterile. Films from the tuberculous foci showed tubercle bacilli. Cultures from the lungs and heart's blood of the control monkey gave *Pneumococcus* Type I.

*Protection Tests.*—The serums of the two vaccinated monkeys were tested for protective substances. Monkey 34 showed a definite amount of protective substance in its serum, the mice receiving 0.0000001 and 0.00001 cc. of *Pneumococcus* Type I both surviving. With the serum from Monkey 35 only one mouse survived—the one receiving 0.0000001 cc. of culture.

This experiment shows that even in the case of the Philippine macaque, a monkey peculiarly susceptible to pneumonia, an adequate immunity against *Pneumococcus* Type I pneumonia may be obtained by the administration of three large subcutaneous injections of *Pneumococcus* Type I vaccine.

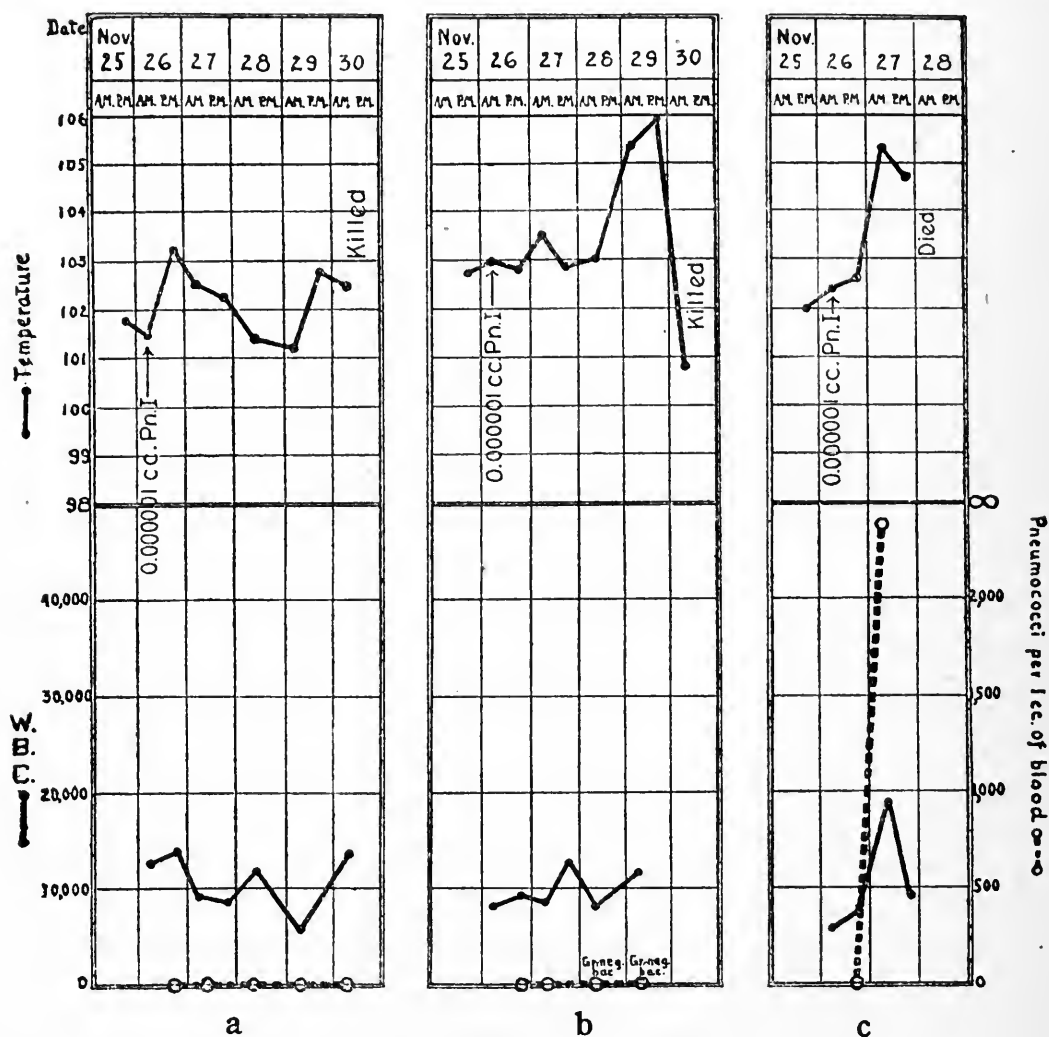
*Active Immunity in Cebus capucinus Following Vaccination with Three Large Doses of Pneumococcus Type I Vaccine.*

A final test of *Pneumococcus* Type I vaccine was carried out on the small South American ringtail. This species was being used for other pneumococcus experiments and it seemed desirable to determine whether an efficient immunity against pneumococcus pneumonia could be obtained in this species by means of three subcutaneous inoculations of pneumococcus vaccine.

*Experiment 5.*—Oct. 29, 1920. Two *Cebus capucinus* monkeys (Nos. 82 and 83) received each 20 billion (0.5 cc.) *Pneumococcus* Type I saline vaccine subcutaneously. Nov. 3. Small nodules at site of previous inoculation. Nov. 4. Each monkey received 40 billion (1 cc.) *Pneumococcus* Type I vaccine subcutaneously. Nov. 11. Each monkey received 60 billion (1.5 cc.) *Pneumococcus* Type I saline vaccine subcutaneously. Nov. 26. Each monkey received 0.0001 cc. of a 24 hour broth culture of *Pneumococcus* Type I intratracheally. An unvaccinated control monkey (No. 86) received the same amount (0.0001 cc.) of culture intratracheally.

The results are shown in Text-fig. 5. Monkey 82 remained lively and well and showed no signs of infection. Monkey 83 remained well until the 4th day following inoculation, when he unfortunately contracted distemper. An epidemic of this disease had broken out among the stock monkeys and, in spite of the fact that the subjects under experiment were isolated in separate cages, Monkey 83 contracted the disease and was quite ill when he was killed on the 5th day following inoculation. Autopsy showed lobar pneumonia of the

right middle and left lower lobes, and *Bacillus bronchisepticus* was obtained in pure culture from the lungs. There was no evidence of a pneumococcus infection at any time during the experiment. Monkey 82 was also killed on the 5th day following inoculation and the



TEXT-FIG. 5, *a* to *c*. Active immunity against *Pneumococcus* Type I pneumonia in Capuchin monkeys following vaccination with *Pneumococcus* Type I vaccine. (*a*) and (*b*) Monkeys 82 and 83; each received 120 billion *Pneumococcus* Type I vaccine subcutaneously. (*c*) Monkey 86; control.

lungs were found free from consolidation. Monkey 86, the control, died with an overwhelming pneumococcus septicemia on the 3d day following inoculation. At autopsy the lungs showed engorgement and patches of incipient pneumonia in the right middle and left lower

lobes, and cultures taken from the lungs and from the heart's blood showed a pure growth of *Pneumococcus* Type I.

*Protection Tests.*—Blood was taken from the two vaccinated monkeys just before the intratracheal inoculations and was tested on mice for the presence of specific protective substance against *Pneumococcus* Type I. Contrary to the usual experience, no protective bodies could be demonstrated in either of the vaccinated monkeys.

This experiment corroborates the results obtained in the previous experiments and shows that in Capuchin monkeys three large subcutaneous injections of *pneumococcus* Type I vaccine confer a high degree of active immunity against the homologous type of pneumonia.

#### DISCUSSION.

The experiments reported in this paper establish a fact which has at least a theoretical importance. They demonstrate that monkeys can be completely protected against *Pneumococcus* Type I pneumonia by means of three subcutaneous injections of *Pneumococcus* Type I vaccine, provided sufficiently large doses are administered. The total dosage of vaccine employed in these studies averages about ten times as large as that used by Cecil and Blake in their unsuccessful attempts to vaccinate monkeys against pneumonia. It may be argued that the dosage employed in the present study could not be made use of in man without exciting severe local and general reactions. It is doubtful, however, whether such large doses would be necessary in the case of man. The average, healthy human being probably possesses more resistance to pneumonia than the average monkey by reason of long continued exposure to *pneumococcus* infection. Furthermore, when monkeys are infected artificially by intratracheal inoculation, a large number of *pneumococci* (400 to 1,000,000) attack the organism at once, whereas, in spontaneous infection in man, the number of *pneumococci* attacking at the onset of pneumonia is probably very small. It would be desirable, however, to administer large doses of *pneumococcus* vaccine to man if the toxic element in the vaccine could be removed without injury to the antigenic element.

The results of the protection tests carried out with the serum of the vaccinated monkeys are in harmony with the results reported in the studies of Cecil and Blake. Protective substances may or may

not be present in the serum of vaccinated monkeys and appear to play little or no part in active immunity to pneumococcus infection in the lung itself.

The intravenous administration of pneumococcus vaccine induces a higher immunity than subcutaneous injection. The intravenous method however would be much more time-consuming and this would be an important consideration where large groups of individuals were to be vaccinated.

#### CONCLUSIONS.

1. The subcutaneous inoculation of monkeys with three large doses of *Pneumococcus* Type I vaccine confers on them a complete immunity against experimental *Pneumococcus* Type I pneumonia.
2. The intravenous inoculation of small doses of *Pneumococcus* Type I vaccine also confers complete immunity against the homologous type of pneumonia.
3. Specific protective bodies may or may not be present in the serum of monkeys vaccinated against *Pneumococcus* Type I. There appears to be no intimate relation between active immunity against pneumonia and the presence or absence of protective substances in the serum of the vaccinated animal.

# DATA ON THE DEVELOPMENT OF *HETERAKIS PAPILLOSA* IN THE FOWL.

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PLATE 18.

(Received for publication, March 31, 1921.)

## INTRODUCTION.

The demonstration that the round worm *Heterakis papillosa* inhabiting the ceca of chickens, turkeys, and certain other birds is a factor in the production of the disease popularly known as blackhead has emphasized the importance of further knowledge of the biology of this worm. Birds other than chickens and turkeys in which it has been reported are the peafowl, guinea fowl, various pheasants, partridge, grouse, quail, bustard, and the domestic duck and goose. Experimental work in the production of blackhead by feeding ova of this parasite had already indicated that its development was in all probability direct. The work presented in this paper was undertaken to determine the course of its life cycle within the host. Artificial incubation and brooding make it possible to rear young birds away from the environment of adults. Thus the problem of control is much simplified and resolves itself largely into one of determining how long ova will remain viable in soil. The danger of wild hosts introducing the parasite will vary with localities. In the locality in which our work has been done this has not proved to be a serious factor, but nevertheless it is one that must be taken into consideration.

Railliet and Lucet<sup>1</sup> and Ackert<sup>2</sup> succeeded in infesting chickens by feeding them developed eggs. The latter author also succeeded in infesting chickens by feeding dung earthworms (*Helodrilus gieseleri*

<sup>1</sup> Railliet, A., and Lucet, A., *Bull. Soc. zool. France*, 1892, xvii, 117.

<sup>2</sup> Ackert, J. E., *Science*, 1917, xlvi, 394.

*hempeii* Smith) taken from a poultry yard. He interpreted the result as indicating that ova had adhered to the worms or were present in the alimentary tract, but did not believe the evidence excluded the possibility that the earthworm might act in some way as an intermediate host.

*Development Outside the Host.*

The fully formed ova are deposited by the female worm in an unsegmented condition in the ceca. They are elliptical in shape and measure 74 to 78 microns long and 41 microns broad. The shell is thick, resistant, and provides a market protection against external conditions. It is fairly uniform in thickness and in a number of eggs ranged from 3.3. to 3.7 microns. It consists of two layers, the inner one frequently of a pale blue color and having a thickness of half or less of that of the outer layer. Between the two layers at one pole is a minute, clear, lenticular body, the significance of which has not been determined. The ova pass out with the cecal contents and undergo development on the ground. The embryo when fully grown lies coiled within the shell and as a rule shows very little tendency to move. The ends of the body are clear and the middle region is marked by a broad column of minute, highly refractive granules. Except in rare instances, to be considered accidental, the developed ova do not hatch until they reach the alimentary tract of a host.

Experiments have been undertaken to determine the incubation period of the eggs, the longevity of the contained embryos, and the resistance of developed and undeveloped eggs to low temperatures and desiccation. These have been conducted in some instances under conditions quite different from those found on the soil, but they serve to give an approximate guide to what takes place in nature.

In observations on the development and resistance of ova both solid and fluid media were used. In the former, ovigerous females were cut up and spread over a layer of salt solution agar consisting of 0.5 per cent salt and 2 per cent agar in Petri dishes. In the latter, worms were cut up and placed in a shallow layer of physiological salt solution. Evaporation was compensated for by adding distilled water. The agar simply provided a moist substratum, with moisture and oxygen conditions somewhat different from those in salt solution cultures.



*Incubation.*—An agar and a salt solution culture were placed in a refrigerator, the temperature of which taken on 7 days ranged from 2.5–8°C. For a period of 24 days the ova failed to undergo development but developed when removed to room temperature. At the same time two similar cultures were placed in a cool room. The temperature was rather uniform and considerably below that of the laboratory rooms. Taken on 8 days it fluctuated between 11.5 and 13.5°C. Development proceeded slowly and it required 38 days for embryos to become completely formed. Eggs on agar and in salt solution developed at the same rate. These observations indicate that the minimum temperature at which ova develop lies somewhere between 8° and 11.5–13.5°C. The slow rate of development at the latter temperatures indicates that they are not far removed from the critical point.

Sets of agar and salt solution cultures were held at room temperature. There was no difference in the rate of development in the two types of cultures. Cultures held at a temperature which taken on 6 days ranged from 19–23.2° C. showed fully developed eggs in 8 days and by the 11th day all had developed. Cultures kept in a somewhat warmer room, in which the temperature taken on 6 days ranged from 22–25.6°C., were developed on the 8th day. In a salt solution culture, in which the temperature observed on 2 days was 18° and 26°C., development was complete in 9 days. Another culture, in which the temperature noted on four occasions ranged from 20–29°C., developed in 7 to 12 days. The development of the ova at room temperature ranging approximately from 18–29°C. therefore required 7 to 12 days.

*Resistance of Ova to Low Temperature.*—To determine the resistance of undeveloped ova to freezing, a salt solution culture was held for nearly 4 days in a refrigerator at a temperature of freezing. When removed to a warm room these eggs developed and produced normal embryos.

An agar and a salt solution culture containing embryonated eggs were placed outdoors in an unheated building for 7 days, from February 27 to March 5, 1920. The maximum and minimum temperature for the period ranged from 5–62°F. The eggs after being removed to a warm room for examination appeared normal and embryos were observed that showed movement.

*Desiccation.*—To study the influence of desiccation on developed and undeveloped eggs, ova were placed on the bottom of an empty

Petri dish and exposed to the atmosphere of a room. From time to time some were removed and placed in salt solution to determine their viability.

Undeveloped ova dried for a period of 16 days during October, 1920, were viable but at the next observation at the end of 41 days were no longer capable of development. At about the same time developed eggs were subjected to desiccation in a similar manner. At the end of 18 days many embryos were alive although a certain percentage had perished. At the next observation on the 49th day all were dead. In another test during October embryos were viable on the 8th day, but on the 10th day appeared to be dead.

*Longevity of Embryos.*—The embryo within the egg has a remarkable longevity. The thickness of the egg shell offers a protection against evaporation and unfavorable conditions.

In two agar cultures made January 23, 1920, and kept at room temperature embryos were observed to be alive as late as July 2 and September 20, respectively. After these dates no observations were made.

In two salt solution cultures, made at the same time as the above and held under identical conditions, on January 25, 1921, after a period of 12 months, although some embryos had perished, most of them appeared normal and movement was observed in certain instances.

A determination of longevity was made under conditions more nearly approaching those in nature. Salt solution cultures were made September 26 and kept at room temperature. At the end of 12 days (October 8), when the embryos were fully formed, the contents of each were transferred to soil in a small flower pot and mixed up with the surface layer. These pots were embedded part way in a pan containing soil and placed outdoors in a cage of wire netting. At intervals the pots were taken to the laboratory, the ova removed and examined with regard to the state of the embryos. It was found that they were still alive after 8 months.<sup>3</sup>

<sup>3</sup> In a field experiment reported elsewhere (Graybill, H. W., *J. Exp. Med.*, 1921, xxxiii, 667), in which young turkeys were exposed to soil that had been unoccupied for a period of 5 months beginning in January, it was found that viable ova were still present.

*Course of Larvæ Within the Host.*

Young chickens were fed the developed ova of *Heterakis* and killed at intervals to determine the location of the larvæ and observe their state of development. The breeds used were White Leghorn, Rhode Island Red, and Plymouth Rock. When introduced into the experiment they were 32 days old. Some controls from the same stock were provided. The cultures were made in Petri dishes by cutting up female worms, collected from chickens, in a shallow layer of physiological salt solution. They were 14 and 27 days old when fed.

The chickens had been reared in incubators and brooders and during the course of the experiments were kept indoors and protected from infestation with *Heterakis*.

The results of the examination of the chickens are given in Table I. The presence or absence of worms in the various situations is indicated by means of positive and negative signs. Unless otherwise stated the method of searching for larvæ consisted in mounting one or more samples of mucus, or contents of regions of the alimentary tract, or scrapings from an organ, and examining the same under the microscope, using a mechanical stage to cover the whole mount. The method used in examining completely any region of the alimentary tract consisted in slitting it open in a mason jar containing water. The jar was then closed and shaken thoroughly to dislodge contents from mucosa and bring them into suspension, after which the organ was removed and washing, sedimenting, and decanting were continued until the supernatant fluid was clear. Then the sediment was placed in Petri dishes and examined under a low power of the microscope. In examining an entire organ such as the liver, it was ground up in a mortar, then suspended in water, and poured through a tea strainer to remove the larger particles. The filtrate was then washed, sedimented, and examined according to the method already described.

The chickens were killed by chloroforming and the examinations made as rapidly as possible. They were killed after periods varying from  $2\frac{1}{2}$  hours to 57 days.

In an autopsy made on a chicken  $2\frac{1}{2}$  hours after receiving ova the eggs were still in the crop and none had hatched. After 24 hours apparently all had hatched. Larvæ were found only in limited num-

TABLE I

No. of chicken.	Period after feeding ova.	Esophagus.	Crop.	Proventriculus.	Gizzard.	Small intestine.	Large intestine.	Ceca.	Cecal wall.	Lungs.	Liver.	Spleen.	Body cavity.	Remarks.
320	hrs. 2½	—	Unhatched	—	—	—	—	—	—	—	—	—	—	Chicken was given no food after receiving eggs.
321	days 1	—	ova.	—	*	—	Many.	Many.	—	—	—	—	—	Organs normal.
275	1	—	—	—	Several.	—	—	+	—	—	—	—	—	Feces passed on autopsy table washed and sedimented. No larvæ or ova found.
322	2	—	—	—	Two.*	—	—	Many.*	—	—	—	—	—	All organs normal.
276	2	—	—	—	—	—	—	+	—	—	—	—	—	" "
277	3	—	—	—	—	—	—	+	—	—	—	—	—	Organs normal except one lung congested.
323	4	—	—	—	—	—	—	+	—	—	—	—	—	All organs normal.
324	7	—	—	—	—	—	—	Many.	—	—	—	—	—	Walls of ceca possibly slightly thickened and contained white specks.
														Mucosa congested. Other organs normal.
278	7	—	—	—	—	—	—	+	—	—	—	—	—	All organs normal except one lung congested.
325	9	—	—	—	—	—	—	+	—	+	—	—	—	Mucosa of ceca congested. Scraping from congested place in lung contained anterior half of larva. Other organs normal.
279	10	—	—	—	—	—	—	+	—	—	—	—	—	Blackhead. All organs except ceca were normal.
280	11	—	—	—	—	—	—	Many.	—	—	—	—	—	Blackhead. All organs normal except ceca.

326	11	-	-	-	-*	-	+	+	+	-	-†	All organs normal except ceca.
281	14	-	-	-	-	-	+	+	+	-	-†	Blackhead. All organs normal except one cecum. Mucosa covered with hemorrhages.
282	14	-	-	-	-	-	+		-†			Blackhead. All organs normal except ceca.
327	16	-	-	-	-*	-	Many.		-§			Ceca showed certain abnormalities but other organs were normal.
285	18	-	-	-	-	-	+		-†		-†	All organs normal.
328	21	-	-	-	-	-	-*		-§		-†	" "
286	23	-	-	-	-	-	+		-†		-†	" "
287	28	-	-	-	-	-	+		-†		-†	Mucosa of one cecum not normal.
288	29	-	-	-	-	-	+		-†		-†	Mucous folds of ceca absent in places.
332	35	-	-	-	-	-	7*					Other organs normal.
335	49	-	-	-	-	-	-					All organs normal.
336	51	-	-	-	-	-	1*					" "
338	57	-	-	-	-	-	8*		-§			" "

Three controls were killed, two on the 17th day and one on the 30th day. The organs were normal. The contents of the ceca were washed and sedimented but no larvæ were found.

\* Contents washed, sedimented, and examined.

† Organs ground up, washed, strained, and sedimented.

‡ Fixed and stained sections.

§ Piece of fresh wall examined microscopically under pressure between two slides.

bers in the posterior part of the small intestine but in large numbers in the large intestine and ceca. After 2 days in one instance two larvæ were found in the small intestine but none elsewhere except in the ceca. The fact that larvæ in one instance were found in great numbers in the large intestine a day after feeding suggests that this is a site where they collect to migrate to the ceca. The point in the alimentary canal where the ova hatch has not been determined. It apparently does not take place in the crop. It seems probable that the small intestine is the site where the embryos emerge. No larvæ were found in lungs, liver, spleen, cecal wall, or body cavity. The larvæ in general had the same character as embryos within the egg.

In summarizing the observations on chickens killed the 3rd day and thereafter, it should be stated that worms were found only in the ceca and in three instances in the walls of the same. In one chicken (No. 325) a fragment of a larva was found in a scraping from a congested lung, but as no others were observed in further mounts, it seems most probable that this had been introduced by accident, possibly on instruments. Examinations in various instances of regions of the alimentary tract other than the ceca, of the lungs, the liver after being ground up, washed, strained, and sedimented, and of washings from the body cavity were uniformly negative for larvæ. Microscopic examination of the washed cecal wall were likewise negative. In two out of the twenty chickens examined after the 3rd day it was not possible to find worms in the ceca. Three controls examined were negative.

A surprising result of the feeding of ova was the light infestation resulting. It is believed that each bird received at least several hundred eggs, yet the number of worms found in certain instances was small. While the worms are still microscopic or visible to the naked eye with difficulty, it is not easy to form an estimate of the numbers present. However, in the case of Nos. 321, 322, 323, 324, 280, and 327, all killed on or before the 16th day, many larvæ were present. The presence of only three larvæ in a cecum of No. 286; two larvæ in the case of No. 287; and seven and eight worms in both ceca of Nos. 332 and 338 respectively, is evidence of the small infestation resulting. There are in all probability a number of factors involved in this result. Among these may be mentioned the artificial method of collecting

and incubating the ova. It is conceivable that this might have a deleterious influence on the vitality of the larvæ. Also the abnormal condition of the ceca and their contents in cases that develop black-head might have an unfavorable influence on the larvæ.

It will be noted that worms reached maturity in 57 days. Taking 7 days, which was observed in experiments at room temperature as the shortest time for embryos to become fully developed, as the minimum period for development outside the body, the entire cycle would require 64 days.

*Development of Larvæ (Figs. 1 to 11).*

Embryos may be freed from ova by pressing on the cover-glass of a fresh mount and crushing the shell (Figs. 1 and 4). If uninjured on emerging they show an active wriggling motion. The maximum width occurs towards the anterior end and the body tapers in both directions, anteriorly to a rather blunt, rounded point, and posteriorly to a long, fine point. The length ranges from 290 to 340 microns, and the maximum width from 14 to 15.6 microns. The anus is situated 45 microns from the posterior end. The anterior end is nude and the mouth is subterminal, being located a little dorsal to the tip of the body. Lateral wings are present, and viewed from the side of the worm they appear as lines, most prominent in the middle region of the body and gradually shading out toward the extremities. The intestine is marked by a band of highly refractive granules extending from a point anterior to the middle of the body to near the anus. The esophagus is slightly bulbous, the bulb being elongated. The cuticula at the anterior tip of the body is slightly thickened.

Larvæ 1 and 2 days old show little change from the embryo just emerged (Figs. 2 and 3). The mouth is still subterminal. The rectum was wedge-shaped and in some instances it alternately expanded and contracted like a pulsating vacuole. A 2 day larva measured 320 microns long and 17 microns broad, and one at 3 days was 338 microns long.

Larvæ at the end of 7 days showed a marked growth (Fig. 7 and same stage as shown in Fig. 8). One measured 0.5 mm. in length and 28 microns broad. Those studied on the 9th and 10th days were somewhat more advanced (Figs. 8 and 9.) One measured 3 mm. In

some instances a pair of small round papillæ were observed for the first time on the anterior end opposite each other.

Larvæ collected on the 14th day were round and smooth on the anterior end with the exception of the presence of the papillæ already mentioned. Five ranged in size from 2.9 to 3.8 mm. A number of rather young larval stages were observed, a circumstance noted also on other occasions, indicating a considerable variation in the rate of growth.

By the 16th day lips were seen forming within at the anterior end, and chitinous structures similar to those of the adult were present in the esophagus or in process of formation. The papillæ already noted on the anterior end were present. These are always seen in optical section of the worm on either side some distance from the mouth, but there may be others not visible on the upper and lower surfaces. At about this time when the lips become free a molt takes place, but specimens showing this have not been encountered. Larvæ measured ranged from 3.2 to 3.9 mm. long.

On the 18th day specimens were found showing further development. Sexes were differentiated, the males being provided with a bursa and papillæ. Larvæ ranged in size from 2.6 to 4 mm. long. On the 29th day worms had reached a maximum of 4.5 mm. in length. By the 35th day some worms were about half grown, and by the 57th day they had apparently reached full development.

#### SUMMARY.

In observations on the development of the ova of *Heterakis papillosa* in cultures, it was found that they failed to develop at a temperature ranging from 2.5–8°C., but developed slowly at a temperature of 11.5–13.5°C. The minimum temperature for development seems to lie between 8° and 11.5–13.5°C. At temperatures ranging in various cultures from 18–29°C. ova developed to their final stage in 7 to 12 days.

Undeveloped ova subjected to a freezing temperature for a period of 4 days were viable at the end of that time. Fully developed ones remained alive when exposed out of doors for a period of 7 days at a temperature ranging from 5–62°F.



Undeveloped ova survived desiccation at room temperature for a period of 16 days, but not for 41 days. Fully developed eggs were alive after desiccation for 18 days, but not after 49 days. In another instance they were no longer viable after 10 days.

Embryos within ova kept in physiological salt solution at room temperature survived during a period of a little over 12 months. Fully developed ova kept in soil outdoors under circumstances approaching natural conditions contained living embryos after a period of 8 months.

From a study of a series of artificially infested chickens killed at short intervals it appears that the ova of *Heterakis* hatch in the small intestine and the larvæ pass by way of the small and large intestines to the ceca where they undergo development to maturity. Larvæ found in the mucosa of the ceca were not in an encysted condition.

Feeding of numerous artificially incubated ova may lead to a light infestation, the cause of which has not been definitely determined.

A period of 57 days was required for larvæ to reach maturity in a host. The entire cycle from egg to adult requires a minimum time of about 64 days.

A brief study of the growth and development of larvæ within the host has been made. No evidence was found of a migration through the tissues. A few penetrate into the mucosa of the ceca.

#### EXPLANATION OF PLATE 18.

Drawings made with camera lucida from fresh material.

FIG. 1. Embryo from an egg in a culture. Magnification about 196.

FIG. 2. Larva, 1 day old. Rendered immobile by adding alcohol and glycerol to mount. Magnification about 196.

FIG. 3. Larva, 2 days old, showing lateral wing. Magnification about 196.

FIG. 4. Anterior end of specimen shown in Fig. 1. Shows esophagus and subterminal position of mouth. Magnification about 834.

FIG. 5. Anterior end of a larva 3 days old. Magnification about 834.

FIG. 6. Anterior portion of body of a larva 4 days old, showing bulbous esophagus and anterior end of intestine. Magnification about 387.

FIG. 7. Anterior region of body of a larva 7 days old. Shows bulbous esophagus. Magnification about 425.

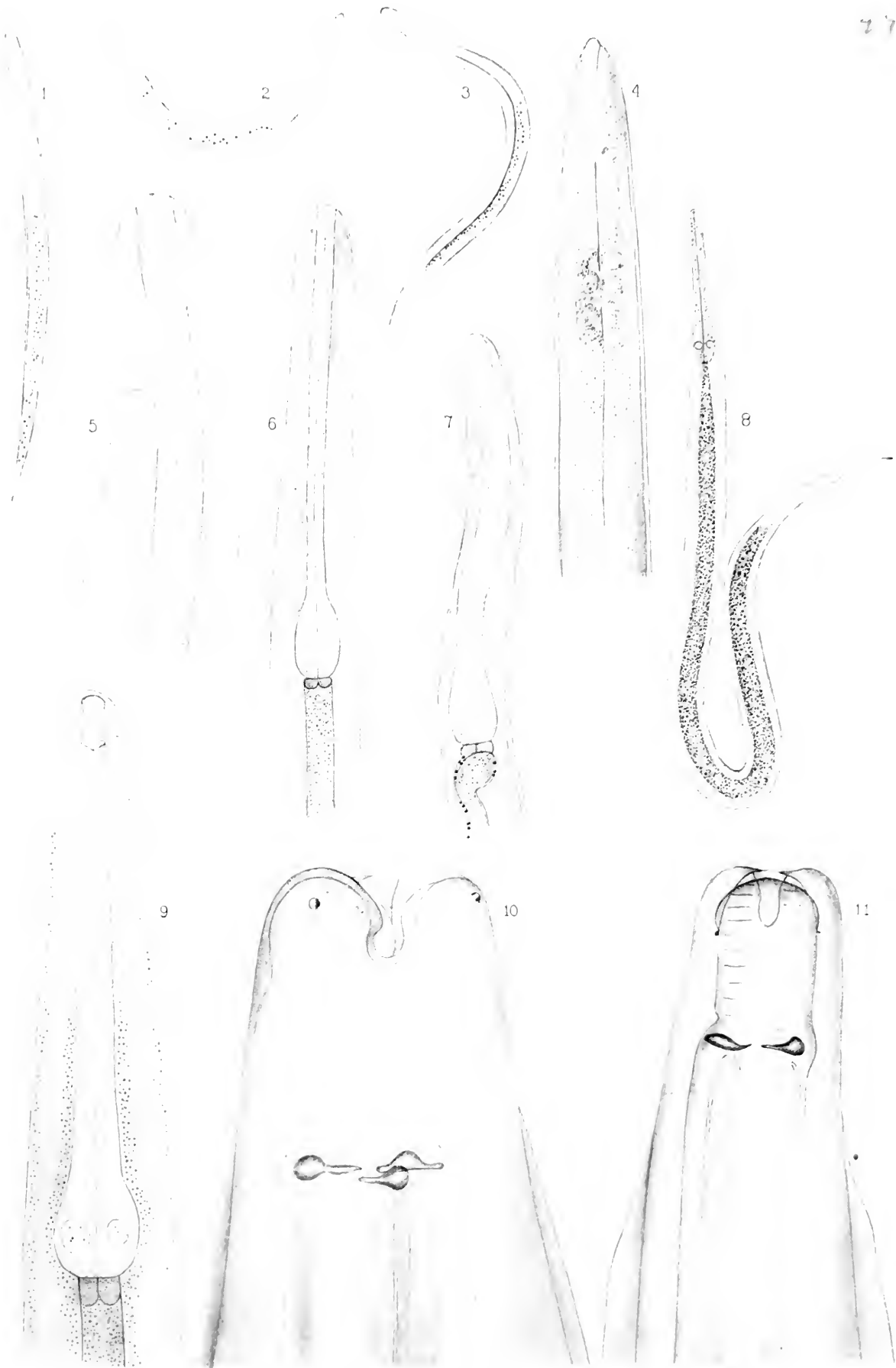
FIG. 8. Larva 10 days old. Shows bulbous esophagus and intestine crowded with granules. Magnification about 46.

FIG. 9. Anterior end of same larva. Shows a differentiation of pharynx. Magnification about 182.

FIG. 10. Anterior end of worm 29 days old. Shows the two ventral lips with teeth and papillæ, the pharynx and chitinous structures of same, and of esophagus. Magnification about 660.

FIG. 11. Anterior end of a worm 35 days old. Dorsal view showing three lips. Magnification about 387.

270



(Graybill: *Heterakis papillosa* in the fowl.)



# LYMPHOPENIA FOLLOWING EXPOSURES OF RATS TO "SOFT" X-RAYS AND THE $\beta$ -RAYS OF RADIUM.

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Murphy and his collaborators have referred to a difference between the effects of large and small doses of x-rays on the circulating lymphocytes of the rat, large doses producing a diminution, and small doses an increase in their numbers (1, 2). The dose required for the latter they refer to as a "stimulating" dose. Our own observations (3) upon the effects of moderately penetrating x-rays, ranging in quantity from very small to quite large doses, showed that, in all cases, there was a preliminary disappearance of lymphocytes from the circulation, generally observable an hour after the exposure to the x-rays. In view of the fact that in many of Murphy's experiments x-rays of a very easily absorbed type were used, it occurred to us that it might be useful to extend our observations with "soft" x-rays similar in character to those used by Murphy, and to supplement them by the use of  $\beta$ -rays of even less penetrating power than these "soft" x-rays. From the data below, it will be seen that, as in our previous experiments, an initial fall in the number of circulating lymphocytes occurs, provided that the blood observations are done soon after the exposure to the radiation.

In view of these findings, it appears that the terms "destroying" and "stimulating" doses as used by Murphy may prove misleading, for they are apt to give the impression that essentially different effects are observed after short and prolonged exposures to x-rays. This, however, has not been our experience, for whereas the lymphopenia following a large dose (4) may continue for as long as a week or 10 days, a small dose produces a lymphopenia which lasts but a few hours.

It is in our opinion important to bear in mind that the lymphocytosis which occurs after a small dose or after a large dose of these radiations follows a primary lymphopenia.

*Experimental Conditions.*

*X-Rays.*—The “soft” x-rays used for our present purpose were those emitted by a Coolidge tube at an alternative spark-gap of 0.75 cm. between spheres 5 cm. in diameter; this is equivalent to  $\frac{3}{4}$  inch between a point-to-point spark-gap. The dose of radiation admin-

TABLE I.  
*Rats Exposed to “Soft” X-Rays for 12”.*

Weight of rat.	Lymphocytes per c. mm. before exposure.	Lymphocytes per c. mm. after exposure.	Fall.
<i>gm.</i>			<i>per cent</i>
118	28,700	10,400	63.6
108	18,400	9,800	46.8
130	27,000	8,800	67.5
About 100.	32,400	12,500	61.4
	30,400	14,300	53.4
	18,000	8,500	55.6
Average.....			58

TABLE II.  
*Rats Exposed to  $\beta$ -Rays for 34 Minutes.*

Weight of rat.	Lymphocytes per c. mm. before exposure.	Lymphocytes per c. mm. after exposure.	Fall.
<i>gm.</i>			<i>per cent</i>
60	5,100	2,700	47
90	8,600	5,900	29
160	8,000	5,200	35
105	19,300	10,400	46
95	13,400	9,100	32
90	7,200	4,500	37
Average.....			38

istered to the rats was equivalent to what is referred to in our paper as a twelve" dose; this corresponds approximately to  $\frac{1}{150}$  of a rad. Our previous experimental finding was that when a rat was exposed to x-rays of a moderately penetrating character for 12", an average reduction of 50 per cent in the number of circulating lymphocytes occurred 1 hour after the irradiation. It will be seen from the data

in Table I that, with an equivalent exposure to "very soft" x-rays, the average reduction in the first six rats selected for the test was 58 per cent.

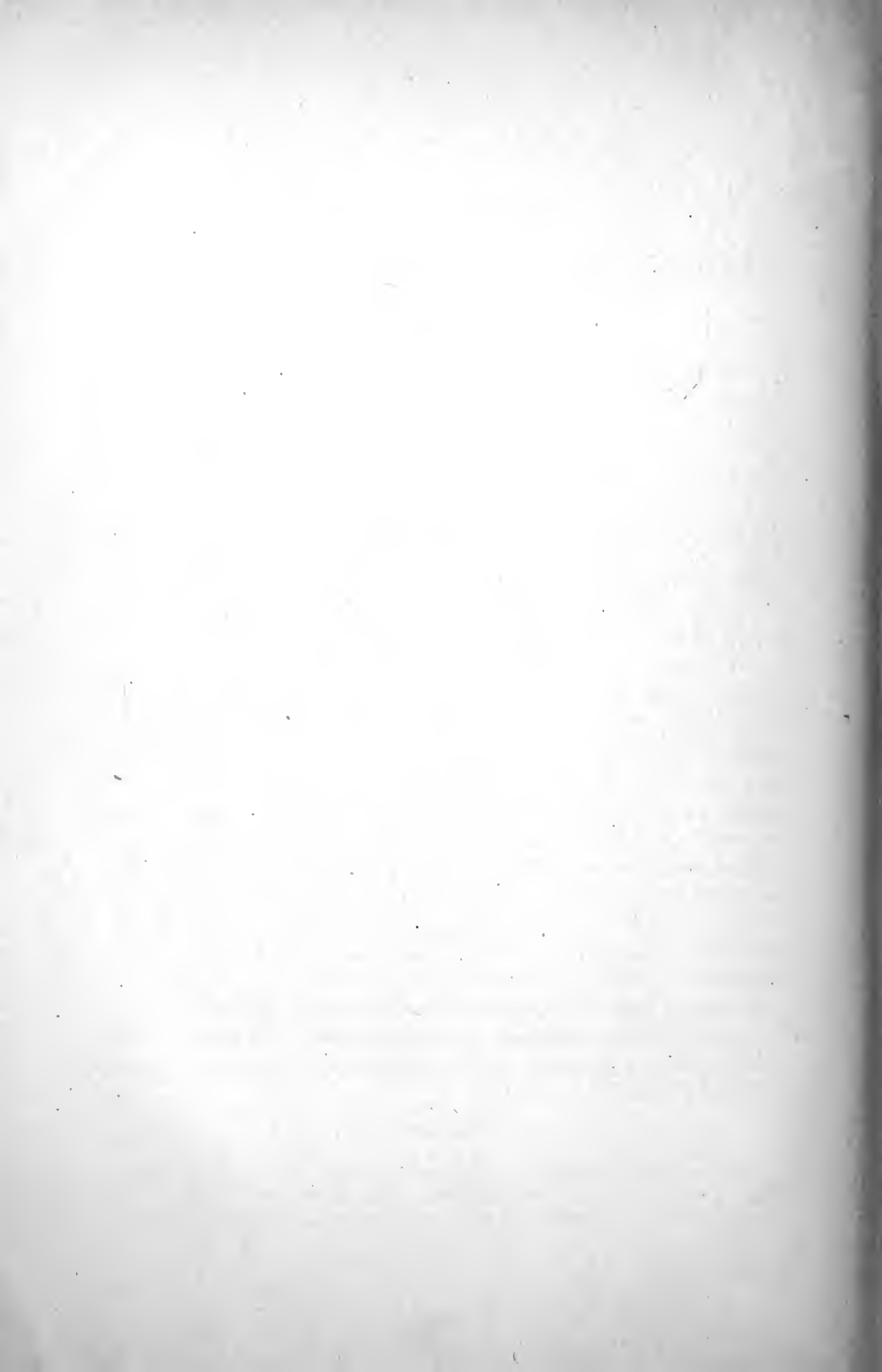
*$\beta$ -Rays.*—Two varnished radium applicators 4 by 4 cm. were used, each containing 80 mg. of radium bromide ( $\text{Ra Br}_2\text{H}_2\text{O}$ ); the animals were confined in a box 15 by 15 by 15 cm.; the radium was let in to the roof of the box, so that it was 16 cm. from the floor, nothing intervening between the radium and the animal on the floor of the box. The exposure lasted 34 minutes, this being the time necessary for the surface dose to be  $\frac{1}{150}$  of a rad, the same as in the x-ray experiment.

Blood examinations were made immediately before the exposures to radium, and again 1 hour after the end of the exposures. Six rats were used; the results will be seen in Table II.

The smaller percentage decrease observed with  $\beta$ -rays is probably to be attributed to their smaller penetrating power than the "soft" x-rays. It seems to us that the experimental results indicate that, had Murphy done blood counts soon after his x-ray exposures, he would have observed this lymphopenia in his small dose studies.

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# THYROIDECTOMY AND PARATHYROIDECTOMY WITH RELATION TO THE DEVELOPMENT OF IMMUNE SUBSTANCES.

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PLATES 19 AND 20.

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An investigation of the literature discloses immediately the unsettled state of the problem of thyroidectomy and parathyroidectomy with relation to the development of immune substances. The reports have been concerned particularly with the site of antibody production. Removal of important organs, preceded and followed by an investigation of natural or acquired antibodies of various kinds, has been resorted to by numerous investigators in the hope of finding one organ in the body which has an undoubted relation to the development of immune bodies, or with a view to prove that no particular one is responsible, and that the phenomenon is entirely a humoral one. Gates performed partial adrenalectomy and studied the development of anti-hen hemolysin, with negative results. Hektoen excised spleen, pancreas, and portions of the liver and intestinal tract. Splenectomy caused diminution in the development of immune substances, while the other procedures had no definite effect. Although thyroidectomy has been performed by a number of investigators on various animals, *viz.*, dog, fox, chicken, horse, and rabbit, yet the relation of the thyroid gland to the development of immune bodies remains an unsettled question because of conflicting results. The relation of the parathyroids in particular to the production of immune substances is still more obscure.

## HISTORICAL.

Hektoen and Carlson worked with dogs, but because of the practical impossibility of removing thyroid without parathyroids, found conditions unfavorable for the study at that time. In 1907, Fassin, working with thyroidectomized

dogs, and rabbits, found a marked diminution of the natural hemolytic as well as the bacterial alexin, but never complete disappearance. Administration of thyroid substance increased the concentration of complement in the serum. This author found that death of the animals followed thyroidectomy rather rapidly, a result not in accord with those of other observers, including ourselves, which leads us to believe that the parathyroids also were removed. Marbé, in 1909, thyroidectomized four dogs and bled them when they developed symptoms. He found a definite diminution of the opsonic power of their serum against staphylococci and tubercle bacilli. He admits that he removed parathyroids as well. He also found an increase in the opsonic power of the serum of a myxedematous patient. In 1910, Fjeldstad, working with rabbits, came to the conclusion that "removal of the thyroids from the rabbit does not, at least during the first month, appreciably affect the formation of immune bodies (more specifically the agglutinins)." Dogs were thyroidectomized by Frouin, and then fed with calcium and magnesium salts. He studied the production of anti-horse and anti-rabbit hemolysin. As a matter of fact, the parathyroids were removed as well. Those animals that developed tetany, some even as long as 14 months after operation, although treated for 3 months with calcium and magnesium salts, showed a diminished production of hemolysin against horse and rabbit blood. Here again it is apparent that parathyroids as well as thyroid were removed.

Launoy and Lévy-Bruhl (1913) worked on five chickens, several of which they thyroidectomized, and in the remainder removed the entire thyroid and part of the parathyroids. They concluded that the thyroid gland plays no rôle either in the resistance of these animals to infection with *Spirochæta gallinarum*, or in the rapidity and intensity of antibody production against this organism. They quote Lerda and Diez to the effect that thyroidectomized guinea pigs were found to be equally, or more resistant to intoxication by tetanus and diphtheria toxins, strychnine, and caffeine than normal animals. However, Houssay and Hug more recently found thyroidectomized horses markedly sensitive to diphtheria toxin. Again, in 1915, Launoy and Lévy-Bruhl conclude that thyroidectomy in chickens does not inhibit the resistance or the antibody production against *Spirochæta gallinarum*. They worked with the protective power of the acquired immune serum against *Spirochæta gallinarum*. In 1915, Hektoen and Curtis thyroidectomized dogs immediately after injecting them with rat blood. Death followed in 10 days—from tetany—a fact which leads one to believe that the parathyroids also were removed. Following the operation no "variation in the usual course and amount of antibody production" was noted up to the time of death.

One of the most recent investigators of the subject is Garibaldi. In January, 1920, he published his results on a study similar to, but not so extensive as ours. He performed thyroidectomy in rabbits, and studied the development of anti-sheep hemolysin. He used seven animals, three of which were controls. It is

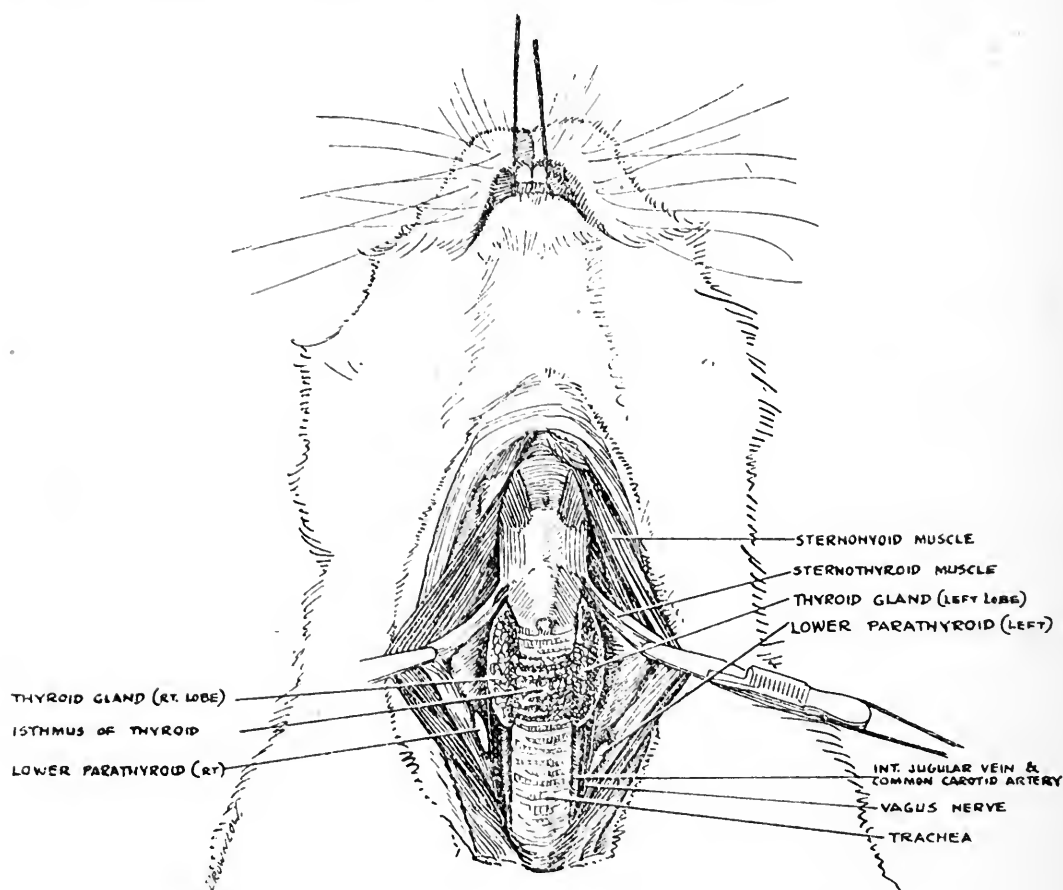
not clear from his paper whether he removed parathyroids as well, nor does he state the concentration of the cell suspension used. The injections were made intraperitoneally and repeated twice at intervals of 7 days. In his titration he used 1 cc. of a 5 per cent suspension of sheep blood corpuscles and a "constant and sufficient quantity of complement." The first and only titration was made 12 days after the last injection—exactly 1 month after the operation. The average titer of the series was 1:7,000 in the thyroidectomized as compared with 1:1,200 in the controls. He comes to the conclusion that the heterohemolytic titer of the serum of the thyroidectomized rabbits immunized during the month following the operation is definitely higher than that of the control animals, and that thyroidectomy seems to favor the formation of antibodies. He leaned for confirmation of his conclusions upon the results of Frouin, and also to a certain extent upon those of Launoy and Lévy-Bruhl. But the latter replied vigorously to this (1920), repeating that their results did not warrant the statement that thyroidectomy favored hyperresistance to infection. Garibaldi's conclusions were based on an experiment with seven animals. The striking feature in his work is the very slight variation and the uniformly high hemolytic titer which he obtained. It is well known that rabbits, normal or otherwise, respond in an extraordinarily variable manner to the injection of sheep blood. A glance at our charts, representing many titrations of the serum of numerous animals will demonstrate the tremendous variations. Some rabbits are very poor producers of hemolysin, the titer of their serum remaining persistently very low, despite frequently repeated injections of blood. This has been the experience of Koopman and others.

Houssay and Hug (1920) recently thyroidectomized young horses. One of the animals immunized to *B. typhosus* gave an agglutinating serum with a titer of 1:50,000 which was considered exceptionally high.

In going over the work of the above quoted investigators we were uncertain in many cases whether they removed thyroid only, or parathyroids as well. The anatomy of the thyroparathyroid apparatus varies in different animals. Since there is good reason to believe that these glands differ in their development and function (Hagenbach), it is reasonable to assume that their removal would have a variable effect, if any, upon the body and its functions in different species. It is important therefore, to know exactly what has been removed. It seems to us that lack of clearness on this point accounts, in part at least, for the variable and often contradictory results obtained. Therefore, a few words on the anatomy of the thyroparathyroid apparatus in the rabbit are not out of place.

*Anatomy.*

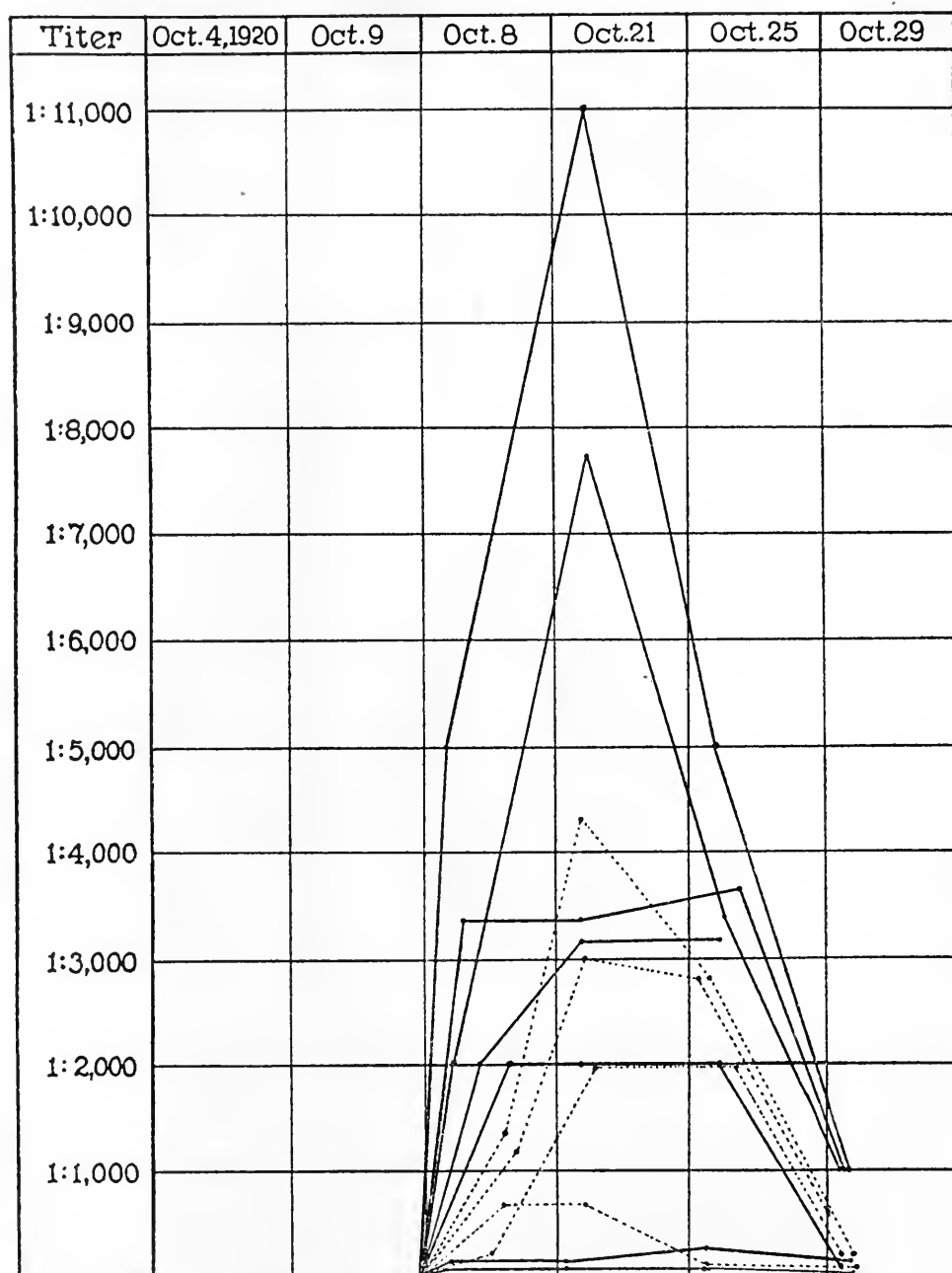
In the rabbit, as in most other animals, the thyroid gland is composed of two lobes and an isthmus (Text-fig. 1). The lateral lobes lie on either side of the larynx and upper portion of the trachea. The tip of the upper pole is hidden beneath the attachment of the sternothyroid muscle to the thyroid cartilage. The isthmus is thin and covers



TEXT-FIG. 1. In this rabbit the upper parathyroid glands were situated within the parenchyma of the lateral lobes and do not show in the drawing.

usually two or three rings of the trachea immediately below the level of the cricoid cartilage. There are, as a general rule, four parathyroid bodies—two upper and two lower. The position of the upper two varies greatly. In some cases they are situated outside the capsule of the thyroid, internal or posterior to the lateral lobes (Fig. 1), while in a great percentage of cases they are situated either within or under the capsule of the thyroid, or are actually embedded in some portion of

the thyroid parenchyma (Fig. 2). In the rabbit, therefore, it is practically impossible, except in an occasional case, to remove the thyroid gland without also removing the upper parathyroid bodies. The



TEXT-FIG. 2. Thyroidectomy with partial parathyroidectomy. Graphic representation of all the titrations in Series 1. — Thyroidectomized rabbits. .... Normal controls.

lower parathyroids are usually easily recognizable as small fusiform bodies approximately 8 by 2 mm. in diameter. Ordinarily they are light pink in color, but sometimes white or red. They are situated under cover of the sternothyroid muscle in intimate relation with the great vessels of the neck. Their position varies from immediately below the tip of the lower pole of the lateral lobe of the thyroid to several centimeters below it—the left one as a rule being situated about 1 cm. below the level of the right (Text-fig. 1).

On account of the anatomical relations of the thyroid and parathyroids, we divided our experiments into three parts: (a) thyroidectomy with partial parathyroidectomy; (b) partial parathyroidectomy alone (removing only the lower glands); and (c) complete thyroparathyroidectomy.<sup>1</sup>

#### *Thyroidectomy with Partial Parathyroidectomy.*

The rabbits used were approximately 6 months of age and averaged 2.2 kilos in weight. In the first series twelve rabbits were used. The natural anti-sheep hemolysin was first determined, and in no case was found to give complete hemolysis in a dilution even of 1:5, with 0.5 cc. of inactivated serum, 0.5 cc. of 5 per cent cell suspension, and two units of complement. Expecting fatalities, we thyroidectomized seven animals and used the remaining five as controls. Of the latter, three were operated upon for exposure of the gland, without removal. All of the thyroidectomized animals lived, but one control died. Other than slight depression during the first few days after the operation, and slight irritability in one or two animals, nothing abnormal was noted in those that were thyroidectomized. The animals were bled and the serum was titrated 24 hours after the operation. No definite change in the natural hemolysin was noted. 5 days after the operation the rabbits had recovered completely. At that time they were given the first intravenous injection of 2 cc. of a 50 per cent suspension of washed sheep blood corpuscles. This was followed 2 days later by an injection of 3 cc. of a similar suspension, and 2 days after that the animals received 5 cc., the final injection. 3 days after the last injection

<sup>1</sup> All operations were performed under ether anesthesia.

tion, and 12 days after the operation, the animals were bled and their serum was titrated for its hemolytic activity. In all our titrations we used 0.5 cc. of a 5 per cent suspension of washed sheep blood corpuscles, two units of complement, and 0.5 cc. of inactivated rabbit serum in increasing dilutions, and incubated them in a water bath at 37°C. for 1 hour. The highest dilution of the serum which then showed complete hemolysis was recorded as the titer. Titrations were repeated in most instances every 2 days until the height of antibody production had been reached and definitely passed. The graphic record of the titrations shows the great variation of the titers of the sera both of

TABLE I.

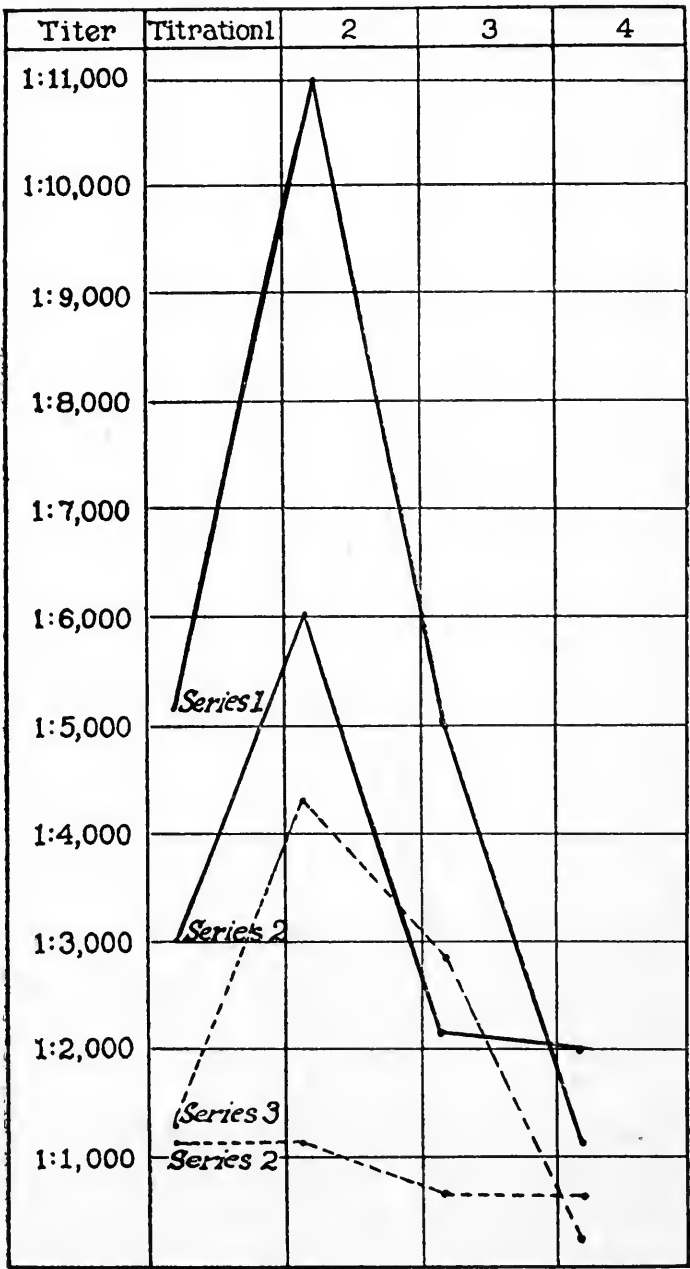
*Thyroidectomy with Partial Parathyroidectomy.*

Titration No.	Maximum titer.		Average titer.	
	Thyroidectomized rabbits.	Controls.	Thyroidectomized rabbits.	Controls.
Series 1.				
1	1:5,200	1:1,400	1:2,500	1:775
2	1:11,000	1:4,400	1:3,850	1:2,450
3	1:5,000	1:2,800	1:2,600	1:1,900
4	1:1,200	1:400	1:575	1:250
Series 2.				
1	1:3,000	1:1,200	1:1,400	1:700
2	1:6,000	1:1,200	1:2,350	1:875
3	1:2,400	1:600	1:1,250	1:350
4	1:2,000	1:600	1:850	1:250
5	1:1,800	1:600	1:700	1:250

the thyroidectomized rabbits and of the controls. The lowest titer of the thyroidectomized rabbits was as low as that of the controls, but in every titration the maximum titer in the thyroidectomized group was considerably higher than the maximum of the controls (Table I and Text-figs. 2 and 3). In the various titrations the average titer of the serum of the thyroidectomized animals was definitely higher than that of the controls (Text-fig. 4).

In the second series in which thyroidectomy with partial parathyroidectomy was performed fifteen animals were used. Three died, leaving seven thyroidectomized rabbits and five controls, in three of

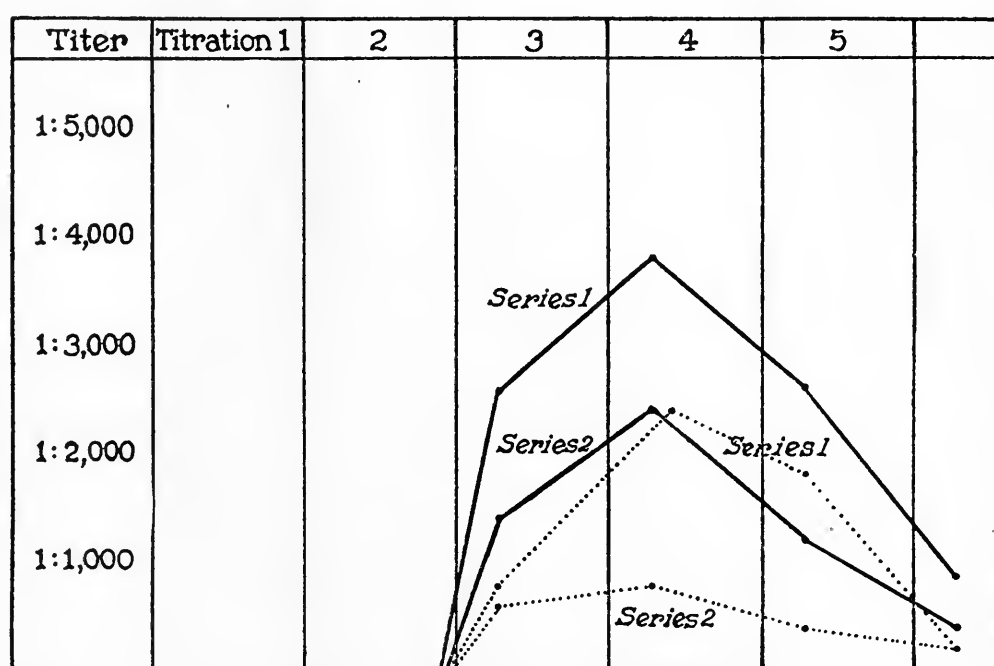
which the thyroid was exposed but not removed. The injections and titrations were performed in the same manner and at the same intervals as in Series 1. The results of the titrations are recorded graphi-



TEXT-FIG. 3. Thyroidectomy with partial parathyroidectomy. Graphic comparison of maximum titers in the various titrations of Series 1 and 2. ——— Thyroidectomized rabbits. . . . . Normal controls.

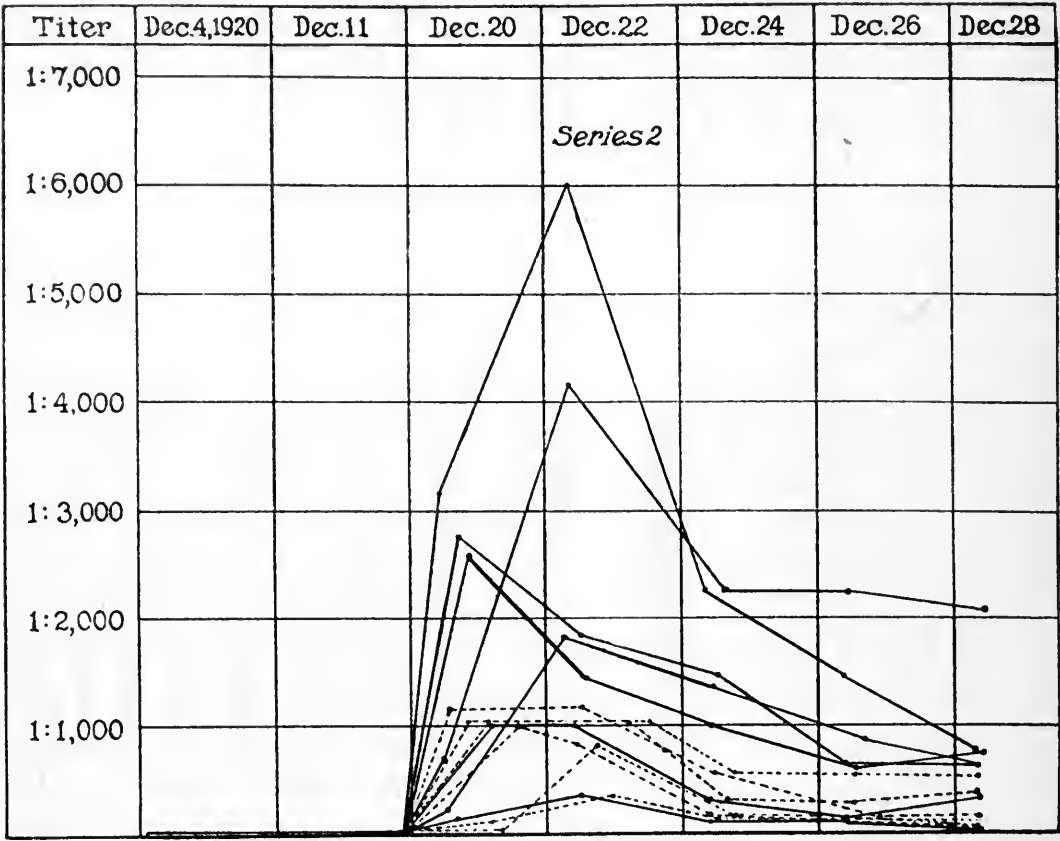


cally in Text-fig 5. Here, again, the lowest titer of the thyroidectomized group was as low as that of the controls, but in every titration the highest titer of the serum of the thyroidectomized animals was higher than that of the controls. A comparison of the average titers of the sera shows that of the thyroidectomized animals was about three times as great as that of the controls (Table I, Series 2). In this series also, the variation in the titer of the different sera was very great, as can be seen in the graphic representation of the titrations (Text-fig. 5).



TEXT-FIG. 4. Thyroidectomy with partial parathyroidectomy. Graphic comparison of average titers in all the titrations of Series 1 and 2. ——— Thyroidectomized rabbits. . . . . Normal controls.

In a third series we repeated Garibaldi's experiment, giving the injections of sheep blood intraperitoneally as he did. We used ten rabbits, five of which were thyroidectomized and partially parathyroidectomized. Not knowing what suspension of sheep blood he used, we washed the corpuscles and made them up to blood volume. In all other respects we repeated his work to a detail. However, the titer of the hemolysin produced was very much lower in our series than in his, and the thyroidectomized rabbits and controls produced



TEXT-FIG. 5. Thyroidectomy with partial parathyroidectomy. Graphic representation of all the titrations in Series 2. ——— Thyroidectomized rabbits. .... Normal controls.

TABLE II.

*Thyroidectomy with Partial Parathyroidectomy Followed by Three Intraperitoneal Injections of Sheep Blood at Intervals of 1 Week (Garibaldi's Method).*

Date of titration.*	Maximum titer.		Average titer.	
	Thyroidectomized rabbits.	Controls.	Thyroidectomized rabbits.	Controls.
1921				
Mar. 12	1:600	1:800	1:350	1:500
" 14	1:1,000	1:1,000	1:450	1:450
" 16	1:800	1:1,000	1:475	1:600
" 18	1:400	1:500	1:300	1:350

\* Last injection Mar. 1. First titration 12 days later.

approximately an equal amount of hemolysin. Only in two of the controls and in one of the thyroidectomized animals did the titer reach 1:1,000 at the height of production (Table II). This part of our work did not confirm Garibaldi's findings.

### *Complete Thyroparathyroidectomy.*

By the above term we mean removal of all grossly recognizable thyroid and parathyroid tissue confirmed at necropsy by a careful investigation of the neck and chest for accessory parathyroid tissue. Naturally enough, serial sections of the neck and chest were not made to rule out possible inclusion of parathyroid gland in other tissues or organs. In a few cases small suspicious bodies were discovered at necropsy low in the front of the neck, but invariably they turned out to be lymph nodes. Four sets of rabbits were employed for this part of the experiment—a total of 60 rabbits being used. Like Carlson and Woelfel who thyroparathyroidectomized foxes, we found complete recovery to occur after extreme symptoms of tetany with violent and prolonged convulsions, although the animals were expected to die. The symptoms developed in less time in our rabbits than in foxes. There was also a tendency to periodicity of the symptoms, and in some the symptoms were delayed, while in an occasional animal they did not develop at all. Other investigators working with various animals report similar experiences. Farner and Klinger, studying the relation between parathyroids and tetany in the rat, found that some recovered even after apparently complete removal. Ferreira de Mira found that guinea pigs may survive complete thyroparathyroidectomy, some living as long as 57 days after the operation. So it would seem that a certain proportion of animals may live after complete thyroparathyroidectomy just as they do after complete adrenalectomy. Whether the explanation is that there are undiscoverable accessory glands, or that other organs take over the function, we are not prepared to state.

Twelve rabbits were used in the first series. Six were completely thyroparathyroidectomized, and of these four survived. The two died of tetany, while the others developed varying degrees of it. Since we did not know how long the animals would live, they were given one intravenous injection of 7 cc. of a 50 per cent suspension of

sheep blood 20 hours prior to the operation. The results of the titrations, which were made at intervals of 2 days, can be seen in Table III, Series 1. All the titers were low in this case, because only one injection was given. A striking difference is seen, nevertheless, between the thyroparathyroidectomized animals and the controls. In every titration the maximum titer reached by the thyroparathyroidec-

TABLE III.  
*Complete Thyroparathyroidectomy.*

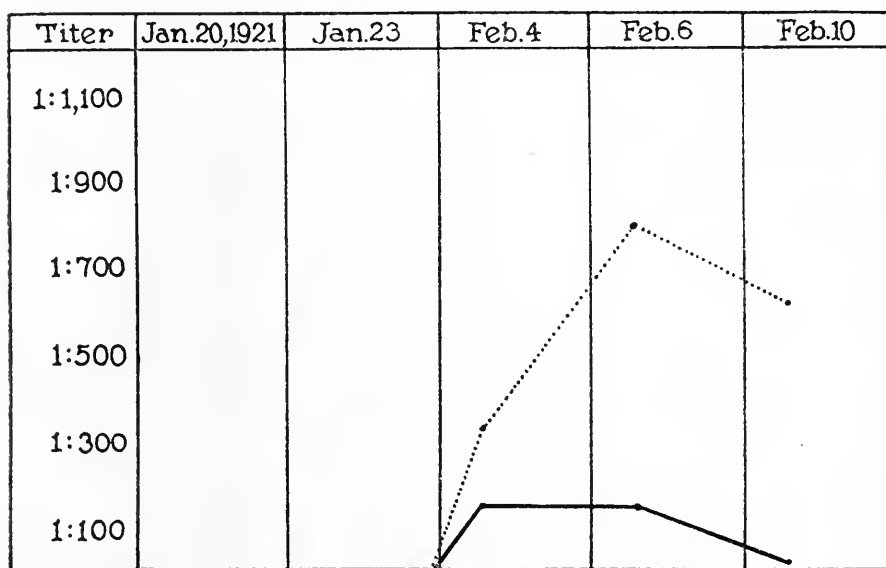
Titration No.	Maximum titer.		Average titer.	
	Thyroparathyroidectomized rabbits.	Controls.	Thyroparathyroidectomized rabbits.	Controls.
Series 1.				
1	1:200	1:400	1:150	1:290
2	1:200	1:1,000	1:150	1:800
3	1:50	1:800	1:15	1:500
Series 2.				
1	1:200	1:2,000	1:150	1:1,500
2	1:400	1:5,000	1:300	1:2,050
3	1:200	1:1,000	1:100	1:600
Series 3.				
1	1:800	1:1,200	1:400	1:725
2	1:2,000	1:6,000	1:1,800	1:3,700
3	1:1,800	1:3,400	1:1,300	1:1,500
Series 4.				
1	1:200	1:1,600	1:120	1:1,100
2	1:800	1:2,600	1:340	1:1,900
3	1:1,000	1:2,200	1:370	1:1,650
4	1:400	1:1,400	1:170	1:1,100

tomized animals was very low compared with that of the controls, and the average titer at the height of production was less than one-fifth that of the controls. A graphic comparison of the average titers in this series is given in Text-fig. 6.

Since some parathyroidectomized animals survived, injections were given in the remaining three series at the same time and in the same

amounts as in the thyroidectomy experiments; *viz.*, 2, 3, and 5 cc. at intervals of 2 days, beginning 5 days after the operation.

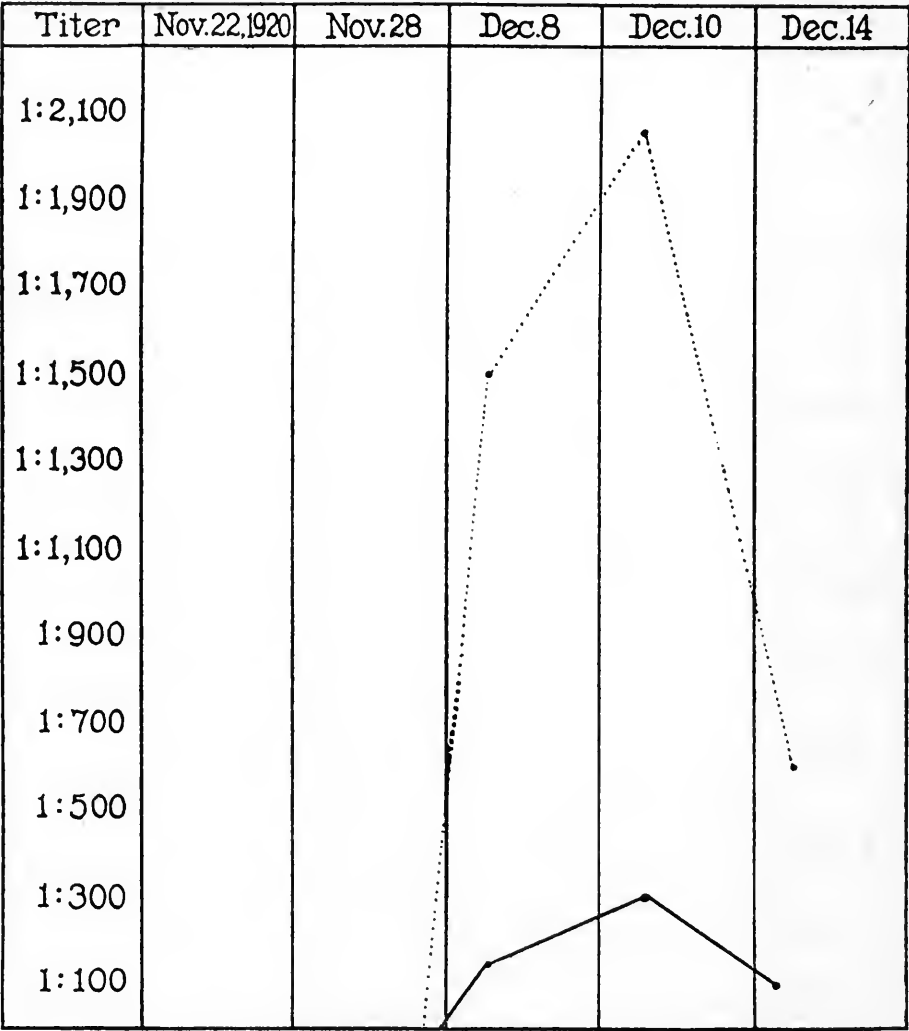
In Series 2, eleven rabbits were completely thyroparathyroidectomized. All developed severe tetany, and only two survived. The latter also had very severe symptoms, but recovered. The titrations in this series also show relatively low titers for the thyroparathyroidectomized animals. In Text-fig. 7 the comparison of the average titers in this series is represented graphically (see also Table III, Series 2). At the height of antibody production the average titer of the thyroparathyroidectomized rabbits was to the controls as 1 is to 7.



TEXT-FIG. 6. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 1. ——— Completely thyroparathyroidectomized rabbits. . . . . Normal controls.

In Series 3, four rabbits were thyroparathyroidectomized and four normal animals were used as controls. In this series we gave several subcutaneous and intravenous injections of a 1.5 per cent solution of calcium chloride in the hope of saving more animals. The same dose was given to the controls also. The animals had severe attacks of tetany, but three survived, and after the third day had no more symptoms. For results of titrations see Table III, Series 3, and Text-fig. 8. Here again there was relatively less antibody production in the thyroparathyroidectomized group, though the difference was not

so striking as in the other two series. In this series the average titer of the thyroparathyroidectomized animals and of the controls was higher than in the others. Whether this is attributable to the administration of calcium we do not know. At the height of antibody pro-

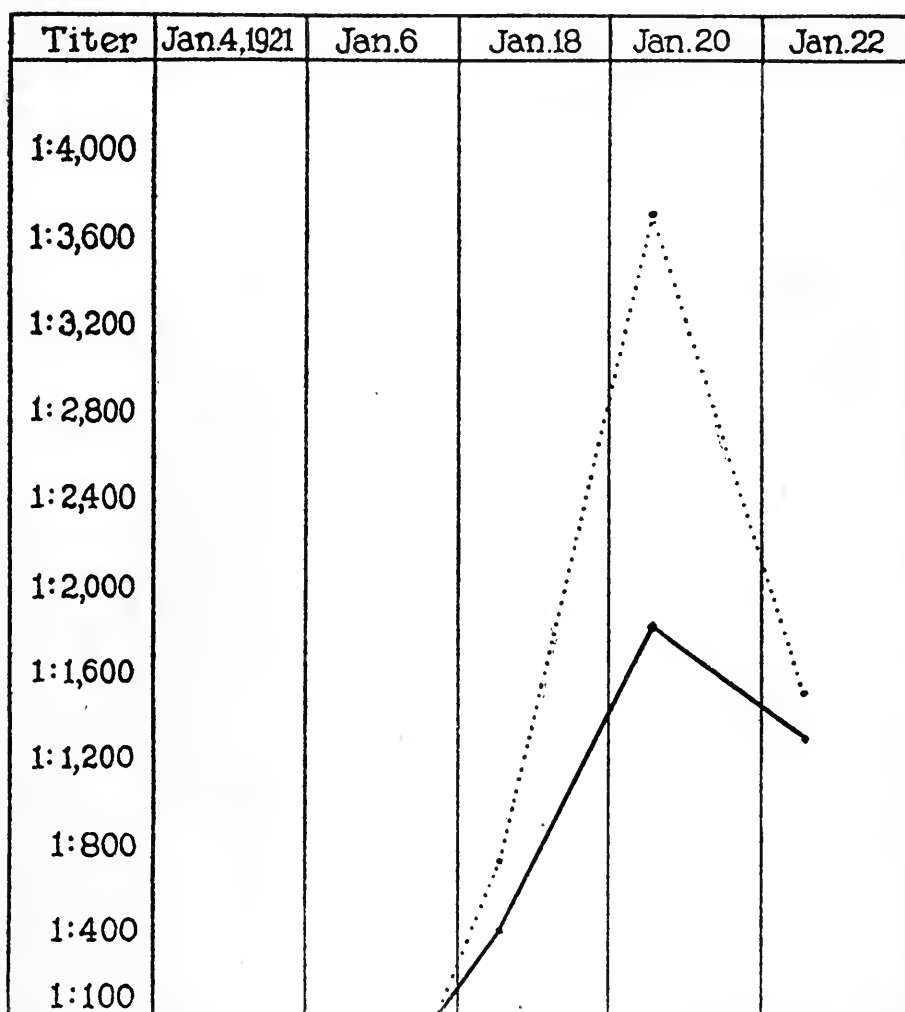


TEXT-FIG. 7. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 2. ——— Completely thyroparathyroidectomized rabbits. . . . . Normal controls.

duction the average titer of the thyroparathyroidectomized rabbits was to that of the controls as 1 is to 2.

In Series 4, eight rabbits had the thyroid and parathyroids completely removed, five had only the lower parathyroids removed, and

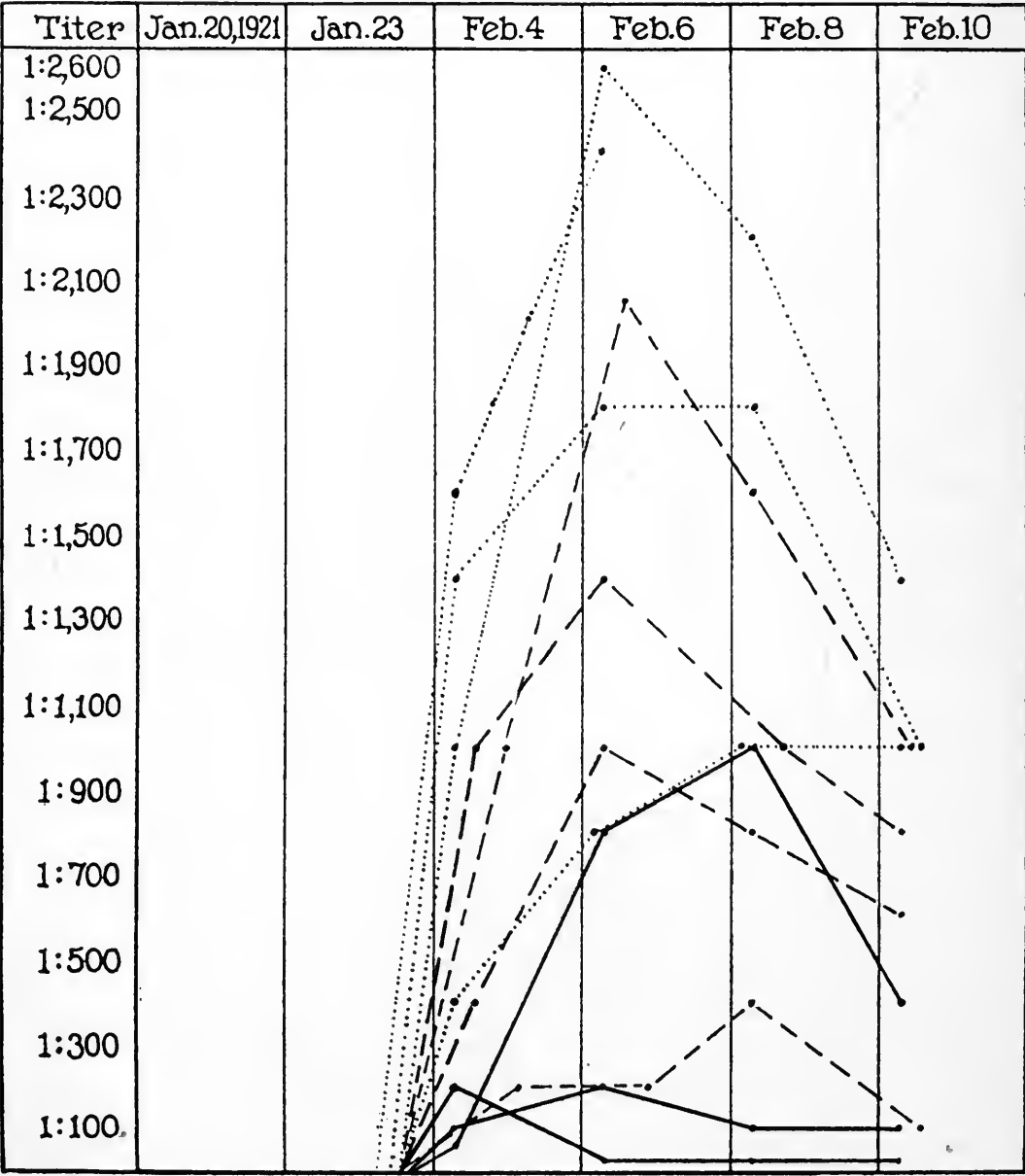
five normal animals were used as controls. In as short a time as 8 hours some of the animals showed definite signs of tetany. Within 24 hours nearly all the animals completely thyroparathyroidectomized developed varying degrees of tetany, and some died. Some received



TEXT-FIG. 8. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 3. — Completely thyroparathyroidectomized rabbits. .... Normal controls.

calcium chloride injections intravenously, but the tetany continued and they died, while other received no injections, had very severe tetany, yet survived. Of the eight thyroparathyroidectomized animals only three recovered. Five more were operated upon 2 days later, and of these only one survived. This one also had very severe

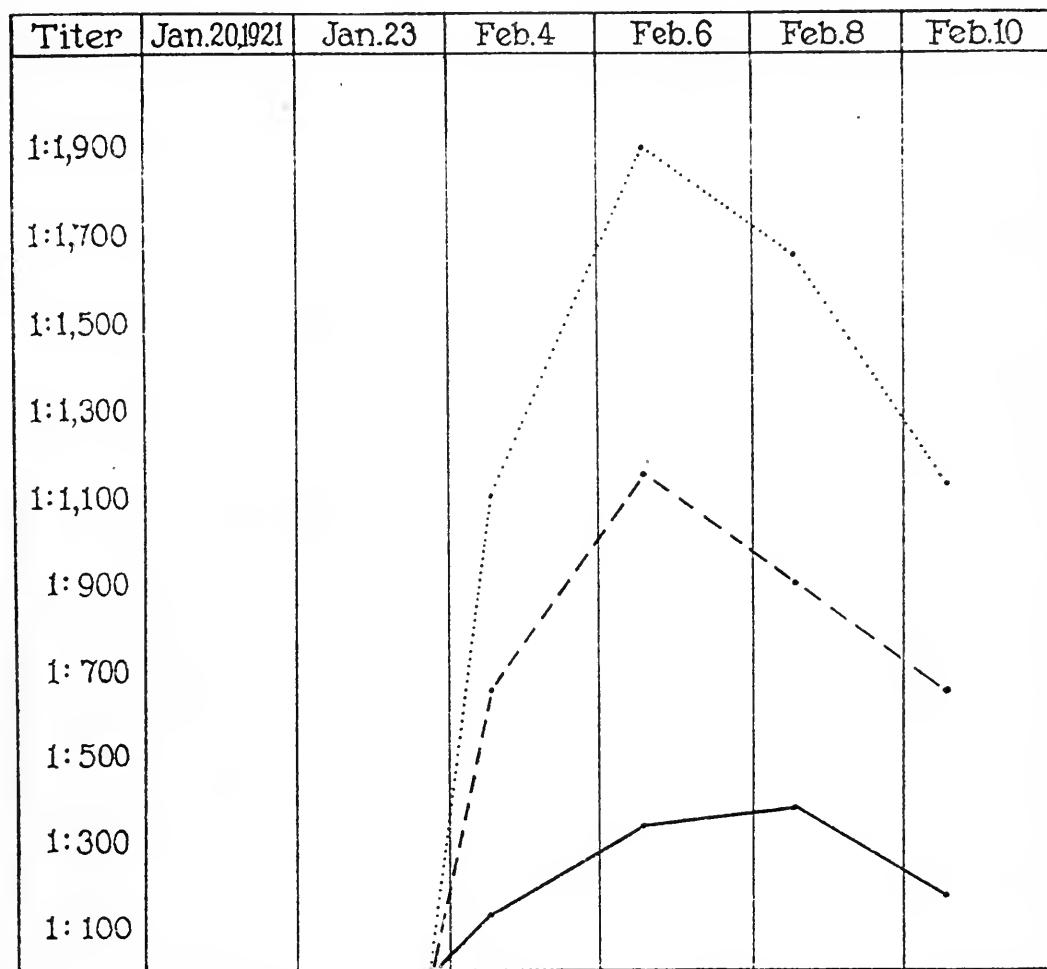
attacks of tetany but recovered and remained well. It was autopsied recently and not a vestige of thyroparathyroid tissue was found. For the results of the titrations, see Table III, Series 4, and Text-fig. 9. Those animals that were partially parathyroidectomized



TEXT-FIG. 9. Complete thyroparathyroidectomy. Graphic representation of all the titrations in Series 4. ——— Completely thyroparathyroidectomized rabbits. ----- Partially parathyroidectomized rabbits. .... Normal controls.



developed a little irritability and depression, but all survived. In this series the completely thyroparathyroidectomized animals developed the least amount of antibodies, while next in order came the partially parathyroidectomized, and finally the controls. Text-fig. 10



TEXT-FIG. 10. Complete thyroparathyroidectomy. Graphic comparison of average titers in Series 4. ——— Completely thyroparathyroidectomized rabbits. ----- Partially parathyroidectomized rabbits. .... Normal controls.

is a graphic comparison of the average titers in the various titrations of this series. The average titer of the completely thyroparathyroidectomized animals was approximately one-sixth that of the controls.

Finally, we took six thyroparathyroidectomized animals from Series 2 and 3, operated upon from 2 to 3 months previously. Three of them were fed with parathyroid tablets (Armour), 0.15 grain daily for a week and were then given a series of three injections, this time of bovine blood corpuscles, 2, 3, and 5 cc. of a 50 per cent suspension, at intervals of 2 days. No difference was found in the development of hemolysin between those upon which opotherapy was practised and the controls, but it is very interesting to note that all six were very low in their development of anti-sheep hemolysin, the maximum titer at the height of production being only 1:200, while in a series of normal animals injected in the same way the average titer was 1:1,000. We have no way of knowing, of course, whether we were really administering parathyroid gland and whether the dose was sufficient to have any effect. We did not give the gland by enteric capsules but gave it by mouth, which allowed gastric secretions to act on it before it was absorbed.

Titration was made by the colorimetric method of the hydrogen ion concentration of the serum of rabbits prior to and after thyroidectomy and complete thyroparathyroidectomy. In all cases this was found to be within normal limits (7.6 to 7.8). Hastings and Murray working recently with the plasma of parathyroidectomized dogs obtained the same result.

#### DISCUSSION.

Hektoen, taking advantage of the work on iodobenzoic compounds by Loevenhart and his associates, tested the effect of the administration of iodoxybenzoate of soda on the production of antibodies. He found that dogs which had received iodoxybenzoate produced anti-goat hemolysin of a higher titer than that of dogs which had received injections of iodobenzoate, and higher than that of control dogs which had not received any injections. He concluded that the oxygen element was responsible for this difference. The thyroid gland is supposed to have a definite relation to the process of internal oxidation in the body. Yet in our experiments the removal of the entire thyroid gland with the upper parathyroid glands did not inhibit the production of hemolysin. In fact, the average titer of the serum of thyroidectomized animals intravenously injected with sheep blood was higher

than that of the controls. In view of the divergent results the question of the effect of internal oxidation on the development of immune bodies remains open.

Is the general disturbance created in these animals responsible for the effect on antibody production? During the first few days of the operation the completely thyroparathyroidectomized rabbits showed signs of much greater disturbance than did the thyroidectomized animals, but by the time the injections were begun the condition of both types of animals was approximately the same, and both gradually developed moderate cachexia. Yet, the thyroidectomized rabbits developed a hemolysin of relatively very high titer compared with the control animals, while the thyroparathyroidectomized animals developed a very low titer hemolysin compared with that of normal controls.

#### SUMMARY AND CONCLUSIONS.

1. After thyroidectomy with partial parathyroidectomy the maximum and average hemolytic titers of the sera of rabbits injected intravenously with sheep blood are equal to or higher than those of normal animals similarly injected.

2. Thyroidectomy with partial parathyroidectomy does not inhibit antibody production. This fact is in accord with the results of Garibaldi, Launoy and Lévy-Bruhl, Lerda and Diez, and others.

3. Thyroidectomy with partial parathyroidectomy does not cause serious disturbance in the adult rabbit. If the operation is performed properly, the animals survive and only moderate cachexia develops in time.

4. After complete thyroparathyroidectomy a small proportion of the animals survive even after developing very severe tetany. Those that recover do not show further signs of serious disturbance, but in time develop a moderate degree of cachexia no greater than that of the thyroidectomized animals.

5. Thyroparathyroidectomized rabbits develop anti-sheep hemolysin of a uniformly low titer—on an average one-fifth that of the controls.

6. Injection of bovine blood into rabbits that survived complete thyroparathyroidectomy from 1 to 2 months previously results in the

production of hemolysin of a uniformly low titer compared with that of normal animals similarly treated.

We owe our sincere thanks to Dr. H. T. Karsner for advice and aid during the progress of this work, and to Dr. Julius M. Rogoff and Dr. J. Lucien Morris for technical assistance.

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#### EXPLANATION OF PLATES.

##### PLATE 19.

FIG. 1. Low power photomicrograph showing parathyroid gland (a) situated outside of capsule of thyroid gland (b).

##### PLATE 20.

FIG. 2. Low power photomicrograph showing parathyroid gland (a) situated within the parenchyma of the lateral lobe of the thyroid gland (b).

<sup>a</sup>  
294-

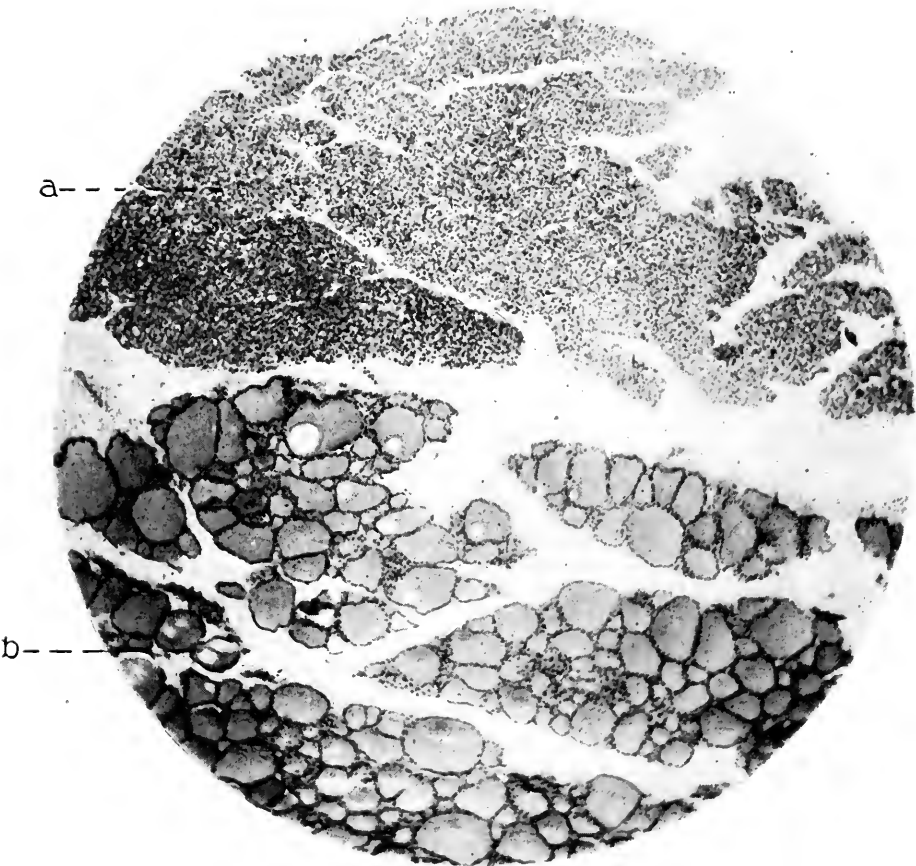


FIG. 1.

(Ecker and Goldblatt: Thyroidectomy and parathyroidectomy.)



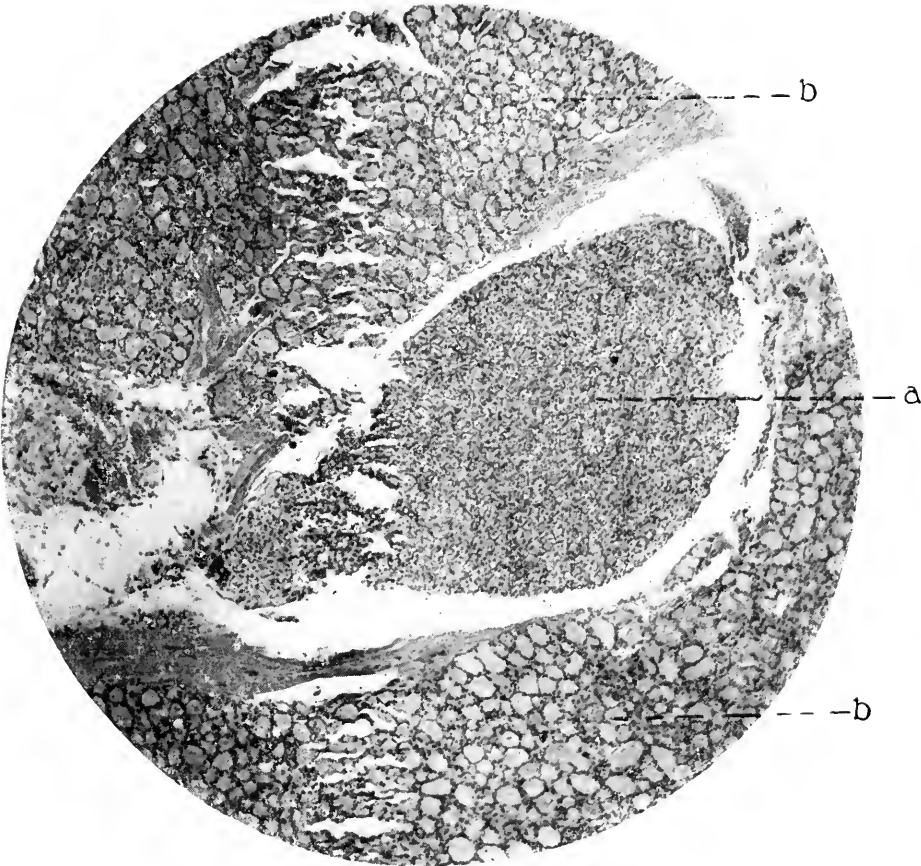


FIG. 2.

(Ecker and Goldblatt: Thyroidectomy and parathyroidectomy.)





# CRISTISPIRA IN NORTH AMERICAN SHELLFISH. A NOTE ON A SPIRILLUM FOUND IN OYSTERS.

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PLATES 21 TO 24.

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In the European lamellibranchiata there have been found a large number of comparatively coarse forms of spiral organisms, provided with a broad, band-like membrane extending from one extremity to the other and winding itself obliquely around the body, one edge being attached to the body, the other free. The organism was first discovered in oysters by Certes (1), who regarded it as akin to the trypanosome and called it *Trypanosoma balbianii*. Laveran and Mesnil (2) considered it a bacterium, while Perrin (3), working under Schaudinn, thought it a protozoon. At that time Schaudinn (4) held the view that the spirochete represented a phase of the trypanosome and that both belonged to the protozoa. The peculiar structure of the organism, as brought out by the technique employed by Perrin, suggested the mitotic and other nuclear figures to such an extent that the organism was accepted as a protozoon by certain protozoologists (5-8). The investigations by Swellengrebel (9) and later by Gross (10) led the latter to place it in the class of *Cristispira*, a terminology accepted by Dobell (11) and Zuelzer (12). The points in dispute are: (1) the interpretation of the membrane; (2) the presence or absence of a periplast; and (3) the presence or absence of a nuclear apparatus, especially the significance of the chambered structure of the body. These questions, which had never been definitely settled, led the writer to undertake examinations of American shellfish. Furthermore, so many species have been described recently in the European shellfish that a comparison from the standpoint of geographic distribution of these cristispiras seemed desirable.

TABLE I.  
*Spirochæta* (Schellack).

Name.	Source.	Length.		Breadth.		Ends.
		Average.	Extremes.	Average.	Extremes.	
<i>Spirochæta balbianii</i> .	<i>Ostrea edulis</i> .	39 $\mu$	35-42 $\mu$	1.3 $\mu$	1.1-1.5 $\mu$	Rounded, no terminal appendage.
" <i>ostrea</i> .	"	41.5 $\mu$	38-42.5 $\mu$	1.1 $\mu$	1.0-1.3 $\mu$	Sharp, " "
" <i>chama</i> .	<i>Chama gryphoides</i> .	45.6 $\mu$	45-46.5 $\mu$	1.4 $\mu$	1.3-1.5 $\mu$	Rounded, " "
" <i>anodonta</i> .	" <i>sinistrorsa</i> .	46 $\mu$	39-50.5 $\mu$	1.0 $\mu$	0.9-1.2 $\mu$	" " "
" <i>spicu-</i>	<i>Anodonta multabilis</i> .	33 $\mu$	28-36.5 $\mu$	0.9 $\mu$	0.7-1.1 $\mu$	Pointed, terminal filament.
<i>lifera</i> .						
<i>Spirochæta modiolæ</i> .	<i>M. barbata</i> .	37.5 $\mu$	36-40 $\mu$	0.8 $\mu$	0.7-0.9 $\mu$	Rounded, no terminal appendage.
" <i>pinna</i> .	<i>P. nobilis</i> .	30.4 $\mu$	29-31 $\mu$	1.0 $\mu$	0.8-1.1 $\mu$	" " "
" <i>lima</i> .	<i>L. inflata</i> , <i>L. hians</i> .	37 $\mu$	35-41 $\mu$	1.4 $\mu$	1.0-1.8 $\mu$	" " "
" <i>cardii</i>	<i>C. papillosum</i> .	19.1 $\mu$	18.5-20 $\mu$	1.2 $\mu$	1.1-1.4 $\mu$	" " "
<i>papillos</i> .						
<i>Spirochæta tapetos</i> .	<i>T. decussata</i> .	34.5 $\mu$	29-35 $\mu$	1.3 $\mu$	1.1-1.4 $\mu$	Rounded, occasionally terminal appendage.
" <i>acumi-</i>	<i>Tapes lata</i> .	47 $\mu$	43.5-49.5 $\mu$	1.0 $\mu$	0.9-1.1 $\mu$	Pointed, no terminal appendage.
<i>nata</i> .						
<i>Spirochæta saxicavæ</i> .	<i>Sax. arcica</i> .	31 $\mu$	30-32 $\mu$	1.7 $\mu$	1.6-1.8 $\mu$	Rounded, " "
" <i>gastro-</i>	<i>G. dubia</i> .	29 $\mu$	Constant.	1.2 $\mu$	1.1-1.3 $\mu$	One end blunt, one sharp, no terminal appendage.
<i>chana</i> .						
<i>Spirochæta pusilla</i> .*	<i>Anodonta</i> , <i>Unio</i> , <i>Lima</i> , <i>Tapes</i> , etc.	13 $\mu$	12-14 $\mu$		0.3-0.4 $\mu$	Sharp, pointed.

\* Bosanquet found a spirochete 10 to 12  $\mu$  in length which he thinks may be identical with *Spirochæta hartmanni* of Gonder or with *Spirochæta pusilla* of Schellack. (No crista?)

Schellack (13), Keysselitz (5), and Gross (10, 14) created a large number of species with only slight morphological variations. To quote an example, Schellack set up thirteen species with certain characteristics, as shown in Table I.

As will be pointed out later, the method of classifying these coarse organisms by their morphological features is subject to error because of the great variations in the terminal portions and in the thickness of body according to the degree of fixation, dehydration, or staining; a difference in these factors may cause the same organism to assume a totally different aspect. Hence the writer has studied these organisms not only in stained preparations but also under the dark-field microscope.

TABLE II.

*Occurrence of Cristispira in the Styles of North American Shellfish.*

Shellfish.	No. of specimens examined.	Styles present.	<i>Cristispira</i> present.
<i>Ostrea virginiana</i> .....	298	128	99 (40 degenerated).
<i>Venus mercenaria</i> .....	110	70	8 (3 " ).
<i>Modiola modiolus</i> .....	97	73	4
<i>Ensis americana</i> .....	24	20	0
<i>Mya arenaria</i> .....	24	24 (hard).	0
<i>Macra solidissima</i> .....	12 (dead?).	1	0
<i>Mytilus edulis</i> .....	42	34	0
<i>Pecten irradians</i> .....	39	34 (hard).	0
<i>Fulgur canaliculatus</i> .....	8	2 (?)	0
<i>Nassa obsoleta</i> .....	30	4	0

The present study deals only with salt water shellfish caught in the neighborhood of Woods Hole during the month of August, 1916, and includes *Ostrea virginiana* (oysters), *Venus mercenaria* (quahaugs), *Mya arenaria* (long neck clams), *Ensis americana* (razor clams), *Macra solidissima* (large sea clams), *Pecten irradians* (scallops), *Mytilus edulis* (mussels), *Modiola modiolus* (mussels), *Fulgur canaliculatus* (winkles, whelks), and *Nassa obsoleta*. The number of specimens of each species examined and the positive findings of *Cristispira* and styles are shown in Table II.

As the tabulation shows, *Cristispira* was found most frequently in the styles of oysters, next in clams, and then in modiolas. No other species examined showed any *Cristispira*. In handling the

styles of these shellfish it was observed that those of oysters, although moderately solid, became liquefied into a viscid fluid upon standing for half an hour at room temperature in a small amount of sea water after removal. The styles of modiolas are much smaller than those of oysters and somewhat firmer; those of clams are much more solid and elastic than either of the others and did not become liquefied on exposure to air and sea water at room temperature. Scallops have large and firm styles which remain little changed after removal. Styles from the other varieties are very small and solid. The physical properties of the styles may be partly or chiefly responsible for the presence or absence of *Cristispira* in these shellfish; apparently the consistency of the styles of oysters offers the optimum conditions for the habitation of the organism.

Another striking fact is that, at least in the case of the oysters, the longer they were kept in a tank (eel pond) or the car (sea) after collection from their original beds, the less frequently they contained styles. In such specimens the presence of *Cristispira* is also less frequent, and in many of them only degenerated forms may be found. It is rather easy to determine the freshness of oysters by examining them for styles and the activity of the *cristispira* contained in them; even freshly collected oysters soon lose the styles if they are not extracted immediately. In one instance 48 oysters which had been kept in the eel pond 24 hours after collection were opened one after another and left at room temperature for about 30 minutes, when they were searched for styles, but unsuccessfully. Six more oysters from the same lot which had not been opened all had perfect styles, and five contained *Cristispira*. These external factors must be taken into consideration in making a survey as to the frequency of *Cristispira* infection of oysters. This rule does not apply to those shellfish whose styles are firm and resistant to manipulation.

The main object of the present work was to study the conditions under which these coarse spiral organisms can be kept alive or cultivated on artificial media, in order that their biological and morphological characteristics might be better studied. Experiments were also made in which the organisms were subjected to the action of various salts, acids, and alkalies in order to obtain an insight into the finer

structure of the organism. Vital stainings with neutral red, crystal violet, brilliant cresyl blue, methyl green, methyl orange, Bismarck brown, and Janus green were also applied.

*Dark-Field Examination of Styles.*

*Oysters.*—The style from the oyster is a glassy, somewhat elastic, cylindrical body of about 2 to 3 cm. long and about 3 mm. in diameter at the thickest portion, tapering to a point at the end. When placed in a Petri dish it becomes a sticky viscid fluid; it dissolves in sea water. When a drop of the liquefied style is examined under the dark-field microscope there are observed in many instances violently motile organisms of large size moving through the field with great velocity. They pass out of the field so rapidly that their exact form is not at once recognizable; they suggest butterflies constantly flapping luminous wings; their movements are backward as well as forward. They become sluggish within a short time and come to rest either in a tortuous form or stretched out. The body is a long cylinder with blunt ends. The end portion is slightly thinner than the rest, but not sharply drawn. There is a thick refractive margin all along the body, indicating the presence of definite peripheral concentration of the protoplasm (perhaps a cell membrane). The inner part of the body seems to present no distinct transverse partitions (so called chambered structure), but there are rows of refractive projections from both sides of the wall, point to point, as if they were imperfect cross-bars; the intervals between the bars are about  $3\ \mu$ . Along the entire body runs a peculiar undulatory narrow membranous structure coiling obliquely and spirally, showing a free edge here and there (known as the crista, or ridge, of the cristispira). It follows a wavy course, and at each wave summit the light is highly refracted. From that point innumerable fibrillar, fan-like radiations run into the body of the organism upon which the other edge of the membranous structure rests or is in connection (Figs. 1 to 3). The fibrils along the free edge of the crista seem to be so arranged as to form a more compact structure than the portion which stretches between the edge and body of the organism. Locomotion appears to be accomplished by alternate bending and relaxation of the undulatory, fibrillar, highly elastic membrane, as is easily demonstrated in a specimen whose

motility is slackening because of exhaustion. In a later period of exhaustion the organism becomes immotile, and the elastic wavy frame of the membrane assumes its full length and shape, causing the body to conform to its spirals (Fig. 8). Only active specimens take the shape of a snake (Figs. 1 to 3). In a freshly extracted style which is still unsoftened by exposure to the air we often find a nest of organisms in one field (Figs. 4 and 5), some assuming positions like coiled snakes, and some rather regularly waved and stretched out. In a few moments the former move rapidly from their resting places with a violent locomotion and swim away; the latter, which represent skeletons or elastic frames (Fig. 8), are degenerated and remain immotile. When a liquefied style containing active specimens of the organism is left for several hours under unfavorable conditions (room) most of the organisms undergo degeneration and are found in various stages from merely stretched out, regularly wavy organisms to skeletons without the body (Figs. 6, 7, and 9). The disintegration of the body is shown by an irregular contour and various degrees of thickness of the body substance. The skeleton often shows clearly its component elastic fibrils, some of which may take the shape of a bushy horse tail (Fig. 6). The gradual overpowering of the body by the elastic membrane in a dying specimen is shown in Figs. 7 and 9.

*Clams*.—The styles of clams are thicker, firmer, and somewhat longer than those of oysters, faintly yellowish, shiny, somewhat opaque, and do not undergo liquefaction in sea water when removed to a Petri dish. For dark-field examination it is necessary to macerate them in a mortar in order to obtain an emulsion. Five only, of 110 styles examined, showed the presence of active *Cristispiræ*; 3 others contained degenerated specimens. The dark-field appearance of the cristispiras found in clams was identical with that of the cristispiras in oysters (Fig. 10).

*Modiolas*.—The modiolas are small mussels, with proportionately small vitreous styles, not longer than 1 cm.; the consistency of the styles is softer than that of clams but firmer than that of oysters. For dark-field examination they can be easily macerated on the slide by pressing under a cover-glass. *Cristispiræ* were present in 4 of 97 styles studied. The organism showed microscopically no distinctive features except that of exceptional length (Fig. 11).

*Examination by Means of Vital Staining.*

The cristispiras from oysters were used for this purpose.

*Neutral Red.*—Within a few minutes the body showed a general brownish yellow coloration, with numerous paired yellowish granules scattered with fair regularity along the entire wall. The membrane, or crista, appeared as bushy brownish fibrils projecting in all directions. The color of the body disappeared in 20 minutes, that of the fibrils in 6 hours. Coiled specimens did not take the stain.

*Crystal Violet.*—The body took a rather deep purple stain, the crista remaining uncolored. Fine protoplasmic reticula with intersecting granules were seen. In some specimens vacuolation of the cell contents occurred, a large mass of substance occasionally protruding into the side (plasmolysis). The deep purple color was still present at the end of 6 hours. There was no chambered appearance of the body.

*Brilliant Cresyl Blue.*—The body took up in mottled fashion a light bluish lavender color. The cross-bars (bluish) of the body were distinctly brought out in a few minutes; the crista remained practically unstained. Complete plasmolysis and decoloration occurred within 6 hours.

*Methyl Green.*—The body stained faint pink without showing any structure; the crista was not stained. Decoloration occurred in 6 hours.

*Methyl Orange.*—There was no coloration. The organisms remained active.

*Bismarck Brown.*—The body became distinctly brownish, with more deeply stained granules throughout the entire length. These granules sometimes occurred in a line along one or both sides of the wall. Degenerated specimens were not well stained. The specimens were practically decolorized in 6 hours.

*Janus Green.*—The body took a deep, dark bluish color, with reticular mottles, the crista a dark blue on its free margin but only a faint blue elsewhere. In some specimens comparatively large round granules of varying size, stained dark bluish, were seen scattered about in several parts of the body. Degenerated organisms did not take the stain. Within 6 hours the color gradually faded.

In general, then, the body of *Cristispira balbianii* took the vital staining, while the crista stained only slightly with some of the dyes and not at all with others. The phenomenon common to staining with neutral red, brilliant cresyl blue, Bismarck brown, and Janus green was the fact that within the first 5 minutes the body of the organism took the stains in such a way as to show the presence of chromophil granules more or less regularly and diametrically paired on both sides of the wall. After a longer period the number of chromophil granules became more numerous, and a sort of reticular distribution of the same substance was brought out, giving the entire body a mottled appearance. In the case of brilliant cresyl blue the cross-bar arrangement of the chromophil substance was distinct but this was followed by complete plasmolysis of the organism, indicating that the chambers were the result of an unfavorable or toxic effect of the dye. Coiled specimens usually showed no deeply staining granules with Bismarck brown or neutral red.

#### *Stained Preparations.*

Several methods were used for the study of the morphological characteristics of the cristispiras, but the best differentiation of the various constituents of the organism was obtained by application of Giemsa's stain and Heidenhain's iron-hematoxylin.

With Giemsa's stain the organisms were variously fixed. In some instances the liquefied styles containing the cristispiras were thinly spread over a clean slide and the moist film surface was immediately exposed to osmic acid vapor (1 per cent) for 1 minute. 30 minutes fixation in absolute alcohol followed. In other instances the moist films were at once immersed in a jar containing Schaudinn's sublimate alcohol, which was maintained at a temperature of 75°C. for 5 minutes and then allowed to cool. Before applying Giemsa's solution the films were rinsed in water, treated first with Lugol's solution, then with 0.5 per cent sodium thiosulfate solution, then thoroughly washed in water. In this procedure the films were never allowed to dry until after being stained with Giemsa's solution. In still other instances the films were first dried in the air, then fixed in methyl alcohol for 30 minutes, then stained with Giemsa's solution.



The object of these various procedures was to study the influence of fixation on the morphology of the organism.

The morphological features revealed by Giemsa's stain were very similar, whether the films were fixed by the osmic vapor or by Schaudinn's hot sublimate alcohol method. In both preparations the body showed a cross-bar structure consisting of deeply stained blue bars alternating with light, almost unstained or faintly pinkish areas. The bars, which were correspondingly wider in larger specimens, appeared somewhat narrower toward the extremities. The relative width of the bluish bar and of the juxtaposed light spaces was very variable in different specimens. In some the width was nearly the same (Figs. 29, 30, and 40); in others the light spaces were much wider than the cross-bars, which then appeared like thin cross lines (Figs. 12, 13, and 31); in still others the reverse was true, in which case a deeply stained bluish body seemed to be segmented by narrow light cross spaces (Figs. 14, 15, 17, and 32 to 34). In the specimens fixed in sublimate alcohol there were specimens showing in some portion of the body several consecutive bars, so thinned out near the middle as to be almost broken (Figs. 13, 17, and 35), the appearance being not unlike that of the organism in the fresh state. The bluish cross-bars were not of uniform width or sharply defined borders, but one end was usually broader than the other, and sometimes a few here and there were placed obliquely to the right angle formed by the wall and the hypothetical axis of the body. In contrast to fresh specimens, the stained organism generally had a tapering form, ending in sharply pointed extremities (Figs. 12 to 14, and 29 to 35). The crista took a brilliant red hue, showing one or more heavily stained marginal fibers, and it ended near the end of the body (Figs. 29 to 35). The connection between the body and the heavy marginal fibers of the crista is so thin that the finer fibrillar structure is seldom brought out. One may encounter in stained preparations specimens caught in full activity (Figs. 12, 14, 15, 17, 19, 29 to 33, and 35) or in the exhausted condition, yielding to the elastic crista, with beginning plasmolysis (Figs. 20 and 21) or with the crista skeleton (Figs. 23 and 36). Sometimes the crista alone may be found (Figs. 37 to 39).

The specimens fixed in methyl alcohol after being air-dried did not show so sharp a structure as those just described. The organism

stained more reddish in general, and the bluish bars were not clearly distinguished (Figs. 16, 18, and 19).

Heidenhain's iron-hematoxylin stain was applied to film preparations of the *cristispira* derived from clams and Mallory's to sections of oyster styles. In the latter the organisms were present in large numbers, usually lying parallel with the course of protein lamellæ, which run concentrically along the long axis of the style like a scallion. Some were coiled, but most assumed wavy forms (Fig. 22). The cross-bar structure was easily distinguished. The crista is occasionally revealed—it is perhaps held closely to the body. (In fresh specimens the crista can always be seen.) Occasionally there are found specimens cut across so that they are visible as a ring. The details of structure are more clearly brought out in the film preparations by adequate differentiation (Figs. 25, 27, and 44). The body is cross-barréd by thin, dark grayish lines, with a more definite contour of the cell wall. The crista is fibrillar and retains a light bluish gray color which is deeper along the marginal fibers. The extremities of the body are not sharply drawn out but are blunt points.

In one of the clams collected in New York Bay in September, 1916, I encountered a rather long variety of *Cristispira*. It was similar in all other respects to the variety met with in the clams studied at Woods Hole. Whether this specimen of *Cristispira* is a new species or merely a variety due to temporary factors has not been determined (Figs. 24, 26, 28, 45, and 46).

#### *Effects of Chemicals.*

The microchemical reactions of various components of *Cristispira balbianii* were studied with a view to gaining an insight into the structure of the organism. Previous investigators have obtained varying results in a similar study of the European specimens. The writer's studies with the American variety were made with the following substances: sodium taurocholate, sodium glycocholate, sodium oleate, saponin, cobra lecithid, acetic acid, hydrochloric acid, sulfuric acid, potassium hydroxide, and ammonia.

*Method of Study.*—One part of the style emulsion containing numerous active specimens of *Cristispira balbianii* was mixed on a cover-glass with one part of the substance, dissolved in sterile sea water;

TABLE III.

*Effect of Chemicals on Cristispira.*

Date.	Chemical.	Concentration.	Result.
1916		<i>per cent</i>	
Aug. 23	Sodium taurocholate.	10	Plasmolysis complete in 5 to 10 min. Skeleton, or crista, resistant. <i>Spirillum ostreae</i> dissolved; bacilli still motile. After 20 hrs. at room temperature skeletons (cristas) showing their fine fibrillar composition are more completely denuded of the cell plasma.
		1	Practically the same as with 10 per cent.
		0.1	Nearly all have undergone plasmolysis; otherwise the same as preceding experiment.
	Sodium glycocholate.	10	Similar in effect to the taurocholate solution.
		1	
		0.1	
	Sodium oleate.	10	This salt produced such a viscid emulsion that no observation was possible.
		1	
		0.1	
	Cobra lecithid.		Almost insoluble in sea water. Observations indecisive.
	Saponin.	10	Complete plasmolysis in 5 to 10 min. Crista not affected but assumes more regularly waved spiral bundles of fibrils (Fig. 9), to which irregularly protruding masses of protoplasm are seen to be attached at some points. <i>Spirillum ostreae</i> and bacilli still motile. After 20 hrs. at room temperature the cristas are still intact. They show their finer structure and their relation to the bodies, which are now mere masses of broken up protoplasm scattered along the regularly undulated spiral cristas.
		1	Same as with 10 per cent.
		0.1	Nearly all have undergone plasmolysis.
	Controls in sea water.		Actively motile for 2 hrs. after being sealed on slides; thereafter gradually became immotile; structure unchanged. After 20 hrs. no degeneration but all in "relaxed" state.

TABLE III—*Concluded.*

Date.	Chemical.	Concentration.	Result.
1916		<i>per cent</i>	
Aug. 24	Acetic acid.	50	After 2 hrs. body smooth, showing cross-bar structure and heavy cell wall; crista shrivelled and indistinct; <i>Spirillum ostreae</i> swollen and indistinct.
		10	Same as with 50 per cent.
		1	" save for the presence of many round bodies suggesting extruded protoplasm of the body of <i>Cristispira balbianii</i> .
	Hydrochloric acid.	50	After 2 hrs. body shows more densely set cross-bar appearance than in acetic acid, but the crista, though perhaps thickened and less distinct than normally, is still attached to the body.
		10	Same as with 50 per cent.
		1	" except for some nodular swelling of the body in parts.
	Sulfuric acid.	50	Similar to results with hydrochloric acid except for more granular appearance of the body substance and less distinctness of the crista.
	Potassium hydroxide.	15	Within 2 hrs. the organisms have practically disappeared, except for a few fragments of the bodies in which no structure save the cell wall can be recognized; no crista found.
		3	Within 2 hrs. the body shows irregularly contoured thick wall with vestiges of thinned out, broken cross-bars; crista seems to be closely attached to the body.
		0.3	Within 2 hrs. there are apparently no changes; body shows distinct cross-bars and heavy cell wall; crista seems to be intact.
	Liquor ammoniæ fortis.	50	Within 2 hrs. no changes; immobilized.
		10	" 2 " " " "
		1	" 2 " " " "
	Controls in sea water.		After 2 hrs. almost all are still active.

the cover-glass was then sealed with vaseline on a slide and examination made under the dark-field microscope. The concentration of each substance and the effects of each upon the organism are briefly summarized in Table III. The observations were made at room temperature (23°C.).

*Survival of Cristispira balbianii under Different Conditions.*

An oyster emulsion containing actively motile cristispiras was used to study the effects of tonicity. The observations were made in hanging drop preparations after 2 hours at room temperature (22°C.). When mixed with distilled water the organisms became immotile, then the cell body assumed a regularly spiral course in conformity with the skeletal crista, presenting at the same time accumulations of a highly refractive substance in round or oval masses along the body at each turn of the spiral skeleton. The phenomenon may be interpreted as due to plasmolysis through hypotonicity. On the other hand, in 10 per cent sodium chloride solution the organisms remained intact, but many coiled forms (Fig. 40) were seen. The body appeared less refractive, but there was no plasmoptysis. In the control preparations made with sea water, most of the organisms became immotile after 2 hours at room temperature; in those kept at 6–8°C. all the cristispiras were still actively motile at the end of 2 hours, and one-fifth to one-tenth after 24 hours; after 40 hours only a few were found to be active in one of the three slides. In dark-field preparations (not hanging drop method), sealed with paraffin and kept at 6–8°C., all cristispiras were disintegrated at the end of 24 hours.

*Attempts at Cultivation.*

Several unsuccessful attempts were made to obtain a culture of *Cristispira balbianii*. The chief obstacles in the work were (1) the lack of bacteriological facilities, and (2) the impossibility of obtaining a bacteria-free suspension of *Cristispira balbianii*.

The culture media employed in the first attempts were made with a filtrate of various styles (oysters and scallops), obtained by passing the sea water solution (or emulsion) of styles (50 styles in 100 cc. of sea water) through a Berkefeld filter V. To 3 cc. of the filtrate were

added, in one set, 1 cc. of 2 per cent glucose agar; in another set 1 cc. of 2 per cent plain agar; in a third set 1 cc. of ascitic fluid; in the fourth 1 cc. of glucose broth; in the fifth 1 cc. of sterile sea water; and in the sixth (controls) 4 cc. of sterile sea water without the style filtrate. All tubes were covered with a thin layer of sterile paraffin oil to prevent evaporation of the culture media. The cultures were set up as follows: All tubes (1 cm. in diameter and 22 cm. high) first received 0.2 cc. of a rich cristispira emulsion from oysters. 3 cc. of the style filtrate were then added to all except the control (sixth) set. The first and second sets were next mixed with 1 cc. of 2 per cent melted glucose agar and plain agar, respectively, the temperature of the agar being about 42°C. at the time of mixing. The tubes were cooled quickly by immersion in the aquarium. The glucose broth and ascitic fluid were added to the other sets and the paraffin oil was finally added to all. Each set was made up in duplicate, one being kept at room temperature and the other at aquarium temperature (about 7°C.).

Examination of the culture tubes was made daily during 8 days, but no growth of cristispira was ascertained. They were brought to The Rockefeller Institute in an ice-packed container and followed for 3 weeks longer, but except in one instance, in which a peculiar large non-motile organism was seen to grow, the results were negative. This organism, which grew in the first set of media (semisolid), bore a close resemblance to *Cristispira*, except that there was no crista. It suggested Gross' *Saprospira*. Whether it was a cristispira modified through artificial cultivation (loss of crista) or an altogether different organism is still a question. Most of the tubes were more or less contaminated with members of the *mesentericus* group.

A spiral organism of oysters, *Spirillum ostreae*, n. sp. (Fig. 43), likewise failed to grow. This spiral organism measures about 8 to 16  $\mu$  in length and 0.5  $\mu$  in the widest portion of the body. Both ends gradually taper off to fine filaments. The number of spirals varies from 4 to 8 and the curves are rather shallow but very regular. The body is elastic but not perceptibly flexible. They rotate rapidly but seldom proceed one way or the other.

In another experiment styles were ground in a mortar with Ringer's or Locke's solutions as well as with sea water and the whole was

sterilized in an autoclave. The opalescent fluids thus obtained were used in experiments similar to those preceding with unsatisfactory results.

The tissue culture technique was employed in order to determine the viability of cristispiras in the presence of various extraneous substances. By careful examination it was possible to collect a dozen styles rich in cristispiras and apparently free from ordinary bacteria. *Spirillum ostreae* was the only variety present and occurred in rather large numbers in some styles. The styles were dissolved in 3 cc. of sterile Ringer's solution; the work was carried out as aseptically as circumstances permitted.

To 1 drop of the style emulsion was added 1 drop of the following liquids respectively: (1) mixture of oyster style-Locke's solution infusion filtrate; (2) oyster broth-Locke's solution-infusion-style filtrate; (3) oyster juice filtrate; (4) oyster broth; (5) Locke's solution; (6) sea water; (7) distilled water; (8) ascitic fluid; and (9) bouillon. Eight Ringer's solution preparations were made as controls.

At the end of 24 hours at 6-8°C. many *balbianii* remained active in three of the eight control (Ringer's solution) hanging drop slides, and in the slide containing ascitic fluid. In all other instances the organisms were inactive and diminished in numbers; there was an increasing mass of bacteria more marked in the slides containing albuminous solutions like oyster-filtrate, Locke's solution-style infusion, and bouillon. At the end of 48 hours only a few cristispiras were still sluggishly motile in the slides containing ascitic fluid and in one of the Ringer's solution controls.

This observation serves to emphasize the difficulty of obtaining a culture of this organism under the conditions of the experiments just described.

#### DISCUSSION AND SUMMARY.

Ten varieties of North American shellfish were examined for the occurrence of *Cristispira* in their styles. A cristispira was found in various numbers in *Ostrea virginiana*, *Venus mercenaria*, and *Modiola modiolus*, but none in *Ensis americana*, *Mya arenaria*, *Mactra solidissima*, *Pecten irradians*, *Mytilus edulis*, *Fulgur canaliculatus*, or *Nassa obsoleta*. Of 298 oysters, only 128 showed the crystalline styles, in

which cristispiras were present in 99. Active cristispiras were found in 59 styles only and degenerated forms in the remaining 40. In 110 clams (*Venus mercenaria*) 70 styles were found, and only 8 of these contained cristispiras; 5 yielded active and the other 3 degenerated cristispiras. In 97 modiolas there were 73 styles, only 4 of which contained cristispiras.

The physical properties of the crystalline styles of these shellfish varied considerably. The styles of the oysters were moderately soft, and when exposed to the air or mixed with sea water they underwent liquefaction, forming a clear, viscid material. The styles from clams and modiolas were opaque and were more firm, not easily crushed even in a mortar. The styles of the scallops were the most solid of all the styles examined. It happened that the softer the styles, the more frequent was the occurrence of the cristispira; in fact, no cristispira was detected in styles other than those of oysters, clams, and modiolas, of which oysters had the softest styles and the largest percentage of cristispira invasion.

The following observations were made regarding the structure of the cristispira found in oysters. The body is a long, flexible cylinder, with blunt extremities, towards which the diameter gradually diminishes. In motion the body rapidly stretches and contracts, forming in the contracted state several serpentine undulations. A membranous appendage (Gross' crista) winds about the body throughout its entire length. The inner margin is in connection with the body, the outer margin is free and is distinctly heavier. The latter is undulatory; that is, the width of the membrane, or crista, is narrower at some points than at others. The membrane is composed of numerous fine fibrils running in a roughly parallel or slightly oblique course, showing interwoven narrow meshes; at the outer margin there is a dense smooth ridge.

The contour of the body is highly refractive, as if possessing a cell membrane. The interior structure, as revealed by dark-field illumination, is an almost homogeneous, less refractive substance, but there are present minute highly refractive granules more or less symmetrically arranged. There is no definite cross-bar or chambered structure. On the other hand, when vital staining with brilliant cresyl blue is applied, there appear numerous paired masses of lavender hue at



fairly regular intervals, suggesting the cross-bar aspect of a stained specimen. In a few specimens there was seen a dim outline of cross-bar effect. Neutral red, Bismarck brown, and crystal violet all bring out deeply stained granules and reticular structure but no definite cross-bars.

While it is difficult to recognize any definite chambered structure in the fresh state, it is very easy to bring out the characteristic banded body by staining the organism with Giemsa's solution. The bands, or cross-bars, which take up a deep bluish color, are of variable width, ranging from a thin line to a square or even oblong block. In some specimens the body is almost blue with thin white cross lines, while in others the blank body is banded with thin bluish bands at irregular intervals. The crista, or undulatory membrane, is stained bright red, particularly along the outer free margin. The fibrillar structure of the crista is usually recognizable. In the specimens stained with Heidenhain's iron-hematoxylin the cross bands, which appear bluish gray, are more distinct and delicate. The bands are not, however, always thin and sharp, but in certain specimens are almost square. The irregularity in the width of the cross bands in various parts of one specimen and in different specimens suggests that the peculiar banded appearance may be a result of fixation and not the natural structure of the organism. At least, no such structure was recognized under the dark-field microscope, which would have revealed such definite septa if they existed. I believe that the cross bands are formed from a homogeneous mass through sudden contraction due to dehydration with absolute alcohol during fixation. In the preparations first dried in the air and then fixed with methyl alcohol this banded appearance is absent. That *Cristispira* has a chambered body, each chamber being equivalent to a single cell, is Gross' interpretation, which is not supported by the observations reported here. Moreover, the protoplasm of the organism readily escapes from the body in distilled water, saponin, or other chemical agents, forming many round masses of extruded protoplasm in different parts of the body. No septa are to be seen in these plasmolyzed or degenerated organisms. Acids, hydrochloric and acetic, failed to bring out any banded structure of the body. It may be deduced that the external surface of the body is a highly refractive cell membrane, and that

within the cell body are homogeneous protoplasm of reticular form and numerous highly refractive granules of varying sizes. Some of the contents take the dark bluish color when stained with Giemsa's solution. In a fixed specimen this particular substance becomes contracted into a series of masses of varying dimension, leaving between each mass a blank space of varying width, and giving a characteristic cross-barred or banded (so called chambered) structure. In some specimens the cross-bars are not seen, but instead a series of broken lines running parallel with the longitudinal axis of the body (Figs. 41 and 42).

The crista is a fibrillar structure, connected with the body at its inner edge. The outer margin is a thickened bundle of fibrils running an undulating course along the entire length of the crista. The crista is elastic and when detached from degenerated organisms assumes a rather regularly wound spiral, consisting of longitudinal bundles of fibrils (Figs. 36 to 38). A fragment of two or three waves may be encountered in a preparation containing many degenerated organisms (Fig. 39). The composition of the crista can best be studied in degenerated remains of the organism. During the life of *Cristispira* it is stretched or relaxed according to the contraction or extension of the body. The elasticity of the crista appears to furnish the organism with a propelling and rotating power upon its extension after being drawn tightly to the body by some contractile apparatus (myoneme) present somewhere within the cell body. The crista serves as a rudder and propeller for the swimming organism. It is interesting to compare here the elastic and regularly waved flagella of certain bacteria and spirochetes; it is possible that the crista of *Cristispira* is a highly modified form of flagella.

The nature of the substance which stains dark blue with Giemsa's stain is not known, but it does not give a chromatin reaction. By Heidenhain's iron-hematoxylin method it takes a dark grayish tint, similar to the cell wall or crista, which are also dark gray. This substance was regarded by Gross and Zuelzer as volutin, which is of nutritive origin. It is probable that there are also embedded within it minute chromidial elements. Multiplication is by transverse fission.

*Cristispira balbianii* is parasitic and does not survive more than a few days in ordinary sea water emulsion, even at its optimum

temperature. In its natural habitat, or the crystalline style, it is usually pure, but is sometimes found in association with a tiny spiral organism (*Spirillum ostreae*). The cristispiras in the styles seem to diminish rapidly when oysters are collected from their beds and transferred elsewhere; oysters kept in tanks or cars for several days do not contain the cristispiras, and in opened oysters the styles disappear promptly at room temperature.

All efforts to cultivate this organism have failed.

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## EXPLANATION OF PLATES.

## PLATE 21.

Magnification uniformly  $\times 1,000$ .

FIGS. 1 to 3. Dark-field view of living specimens of *Cristispira balbianii* from an oyster. Photographed while they were temporarily at rest. The smooth wavy body with sharp contour along the wall, and a somewhat hazy alveolar content are well shown. The undulating crista loosely attached to the body of the organism can be seen. What appear like pointed sticks attached to the organism in Figs. 1 and 3 are the spirillar organisms found in fresh oysters which were merely entangled in the crista.

FIGS. 4 and 5. Dark-field view of *Cristispira balbianii* in the styles of oysters. In the styles there are different forms undoubtedly representing various stages of the life of the organism. In Fig. 4, there are one degenerated and two well preserved specimens, while in Fig. 5 there are also two intact and one long skeleton of a degenerated organism.

FIGS. 6 to 9. Different phases of degeneration of *Cristispira balbianii* due to unfavorable conditions or to age. Fig. 9 shows the protoplasm bulb accumulated near the middle of the body under the influence of a concentrated solution of saponin. Figs. 6 and 8 show the elementary fibrils which constitute the crista.

FIG. 10. Dark-field view of a cristispira from the style of a clam. In every respect this organism is comparable to the *balbianii* found in oysters. It may be the same as that described by Dobell as *Cristispira veneris*.

FIG. 11. Dark-field view of a cristispira from the style of a modiola. Generally somewhat longer than the *balbianii*; otherwise appears identical.

## PLATE 22.

Magnification uniformly  $\times 1,000$ .

FIGS. 12 to 15 and 17. *Cristispira balbianii* from oysters, stained with Giemsa's solution after osmic acid or sublimate alcohol fixation. They are all well preserved.

FIGS. 16, 18, and 19. *Cristispira balbianii* stained with Giemsa's solution after methyl alcohol fixation. All except Fig. 16 appear in good condition, although no definite cross-bar structure can be distinguished.

FIGS. 20, 21, and 23. *Cristispira balbianii* in process of degeneration. Fig. 20 shows the body still solid, almost intact, with a regularly wavy crista. In Fig. 21 the body is still fairly well preserved, but it follows the regular waves of the crista, indicating the absence of any resistance to the form of the crista. Note also the protruding mass of protoplasm near the middle of the body. In Fig. 23 there is only a shadowy trace of the body and the spiral elastic skeleton, or crista, is distinct.

FIG. 22. A section of the oyster style fixed in sublimate alcohol and stained with Mallory's iron-hematoxylin. The organisms show the chambered structure, but no crista can be distinguished.

FIGS. 25 and 27. *Cristispira balbianii* in films from fixation sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

FIGS. 24, 26, and 28. *Cristispira* from a clam caught in New York Bay. The film was fixed in sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

#### PLATE 23.

Magnification  $\times 1,250$ .

Drawings from film preparations fixed in osmic acid vapor and absolute alcohol and stained with Giemsa's solution.

FIGS. 29 to 42. Different specimens of *Cristispira balbianii* showing the general chambered or cross-barred appearance of the body and undulatory membranous appendage, called the crista by Gross. The blue-stained substance which constitutes the cross-bars, or partitions, is seen to be regularly or sometimes irregularly distributed in amount or distance. In some, the substance may not be attached to the wall at all, but forms longitudinal rods of varying lengths as seen in Figs. 41 and 42. In such instances there is no suggestion of a chambered structure.

FIGS. 36 to 39. The final steps of degeneration of *Cristispira balbianii*. Fig. 36 alone still shows the degenerated body while the others show nothing but the fibrous skeletons, or cristas, of the organism. Fig. 39 is a fragment of the crista.

FIG. 43. The spiral organism found in the crystalline styles of fresh oysters caught in Woods Hole Bay.

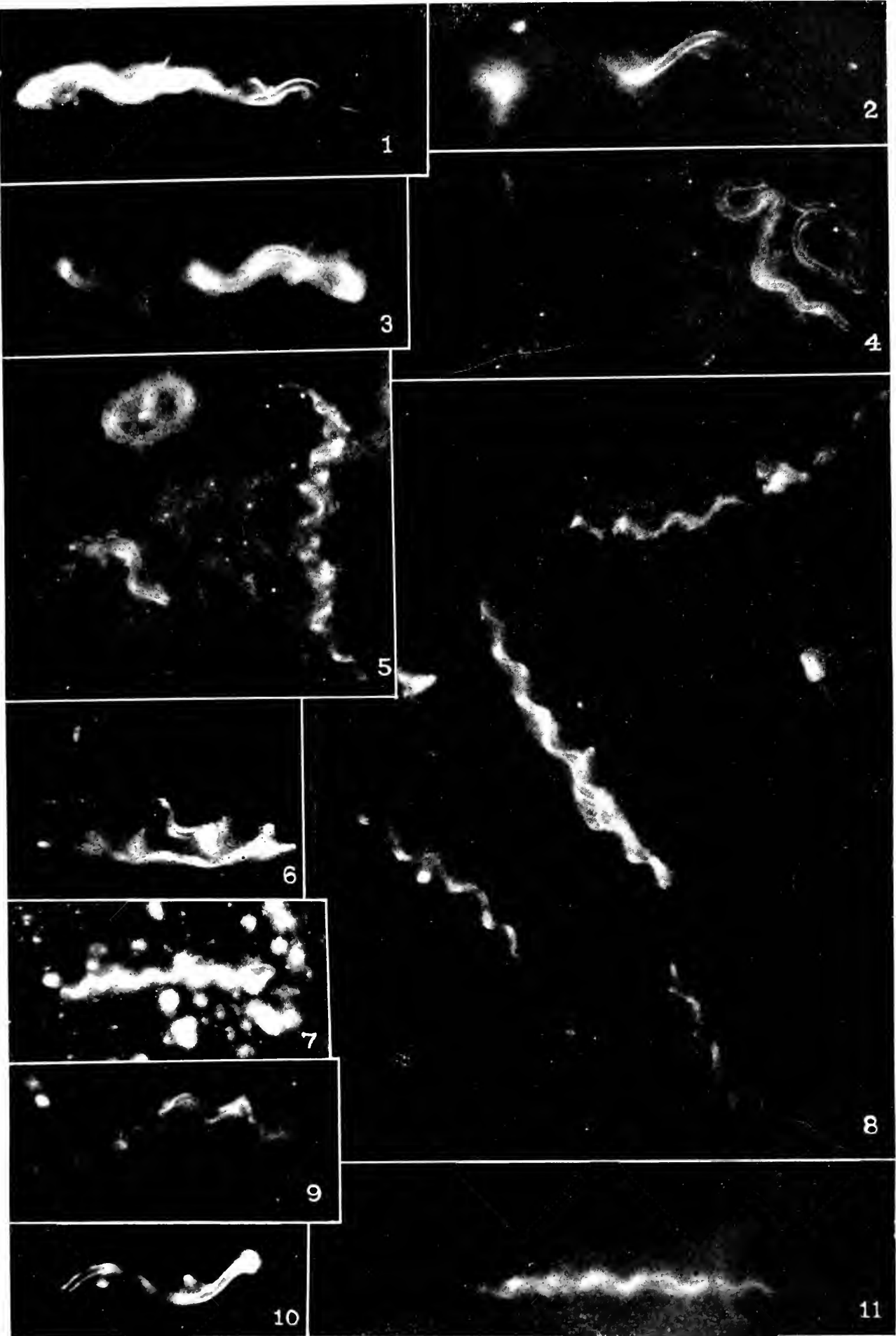
#### PLATE 24.

Magnification  $\times 1,250$ .

FIG. 44. *Cristispira balbianii* from an oyster. Film preparation, fixed in sublimate alcohol and stained with Heidenhain's iron-hematoxylin.

FIGS. 45 and 46. A cristispira (*Cristispira veneris?*) from a clam collected near New York Bay. Sublimate alcohol fixation and Heidenhain's iron-hematoxylin.

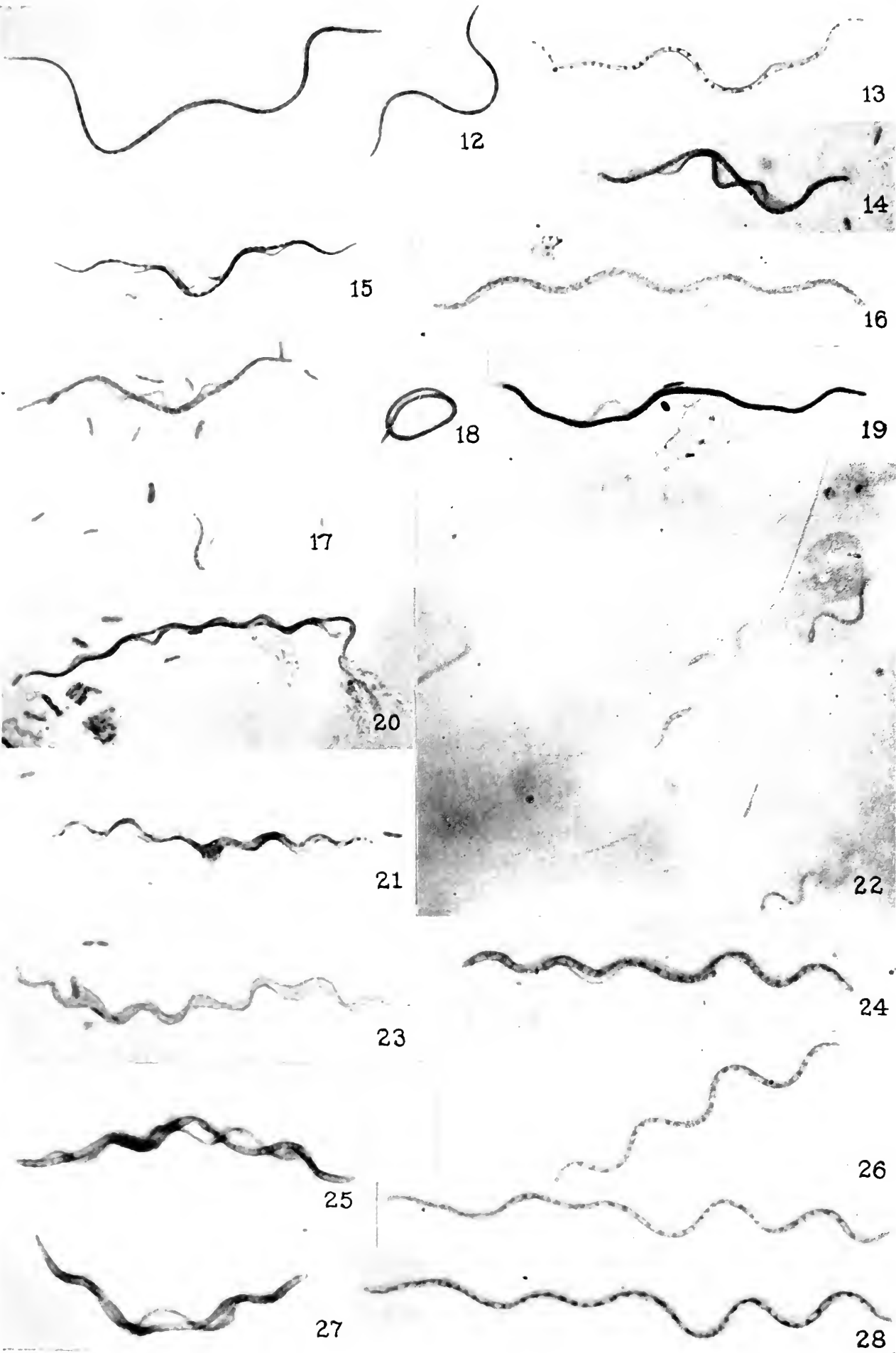
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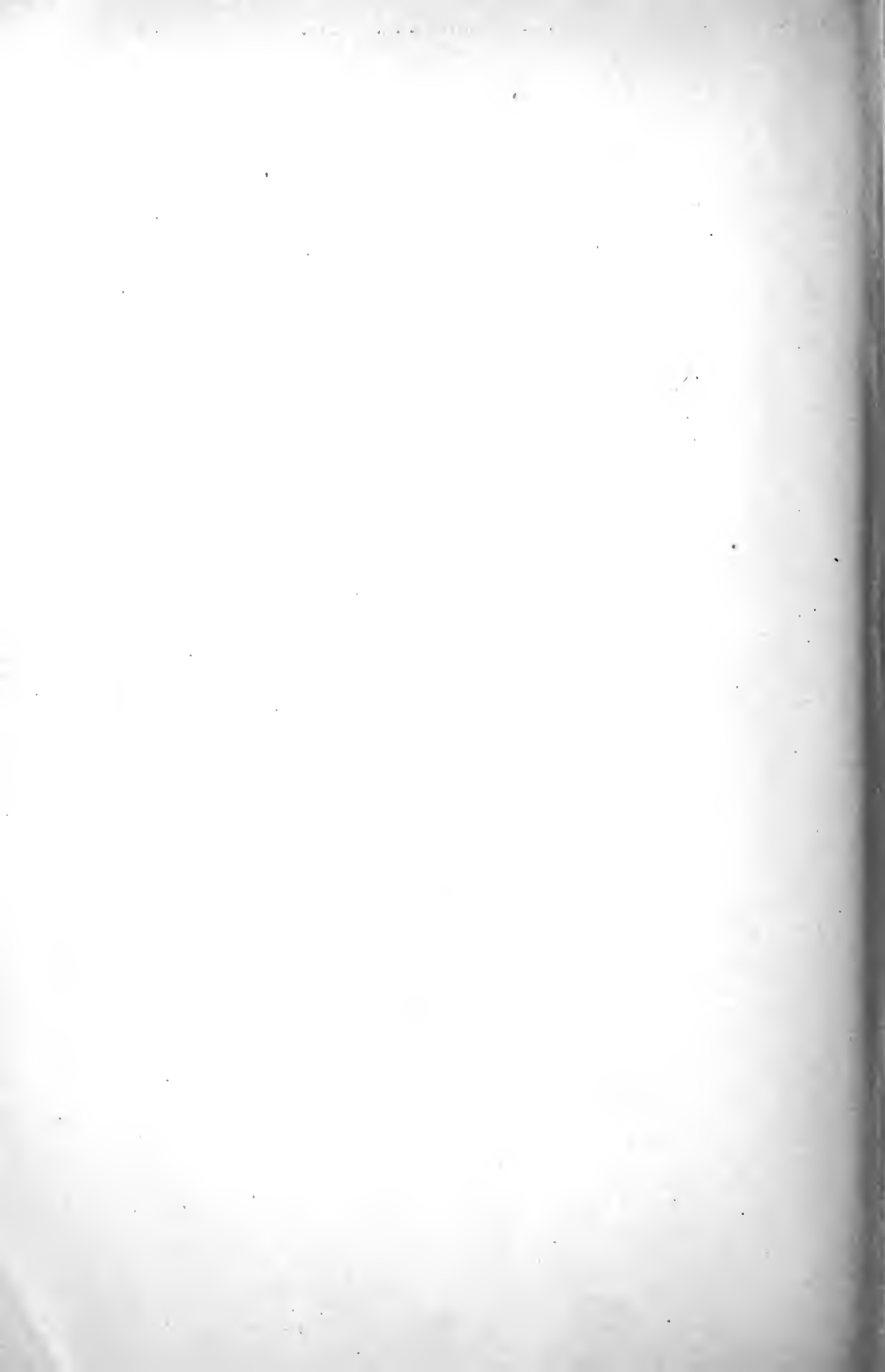
(Noguchi: *Crisispira* in North American shellfish.)

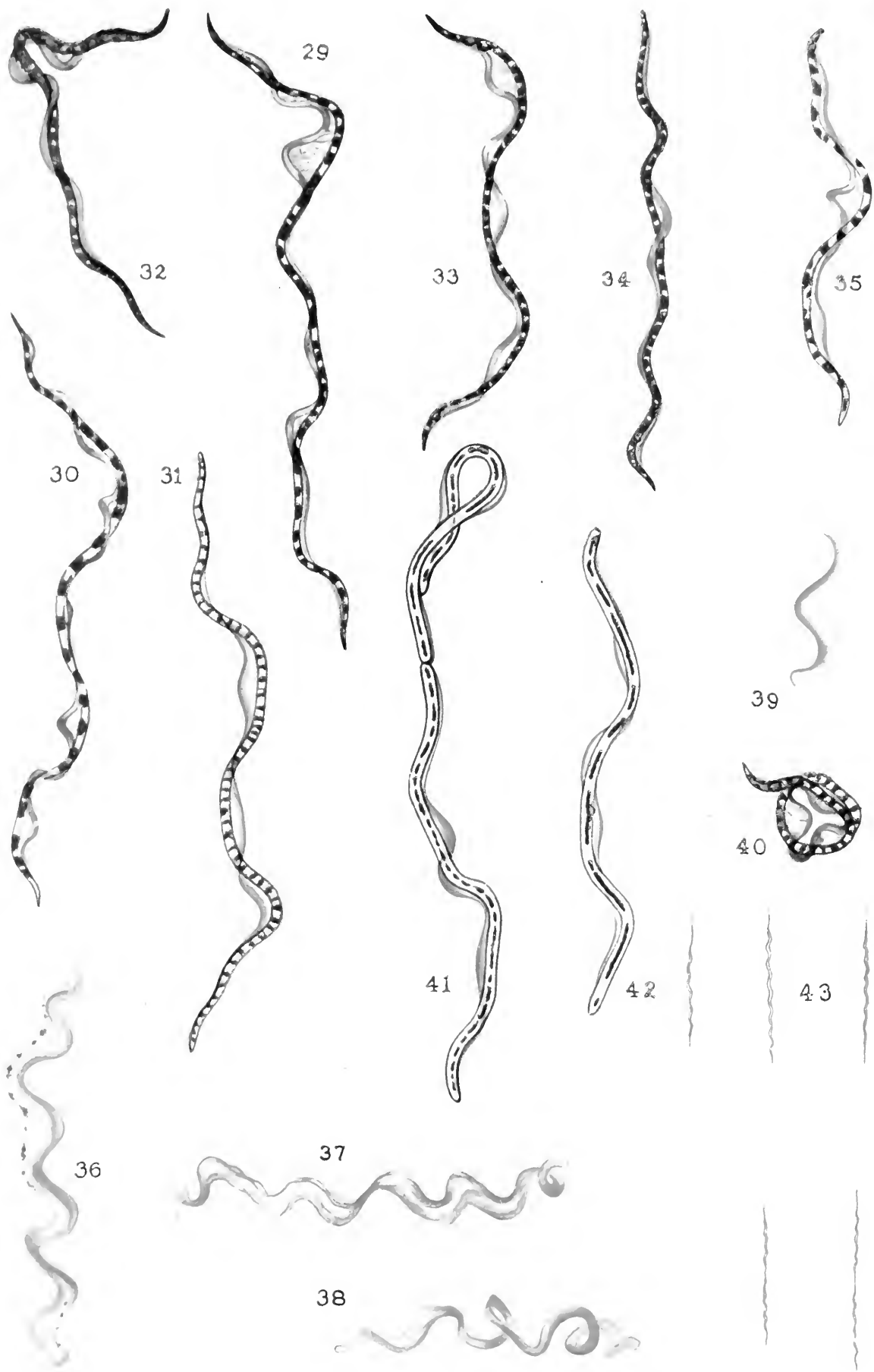






(Noguchi: *Cristispira* in North American shellfish.)



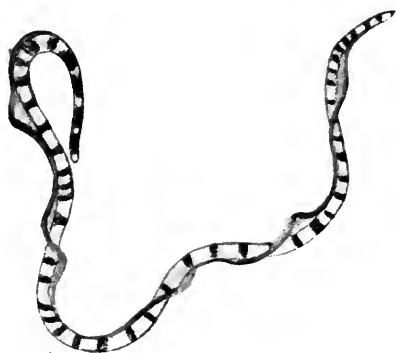


(Noguchi: *Cristispira* in North American shellfish.)





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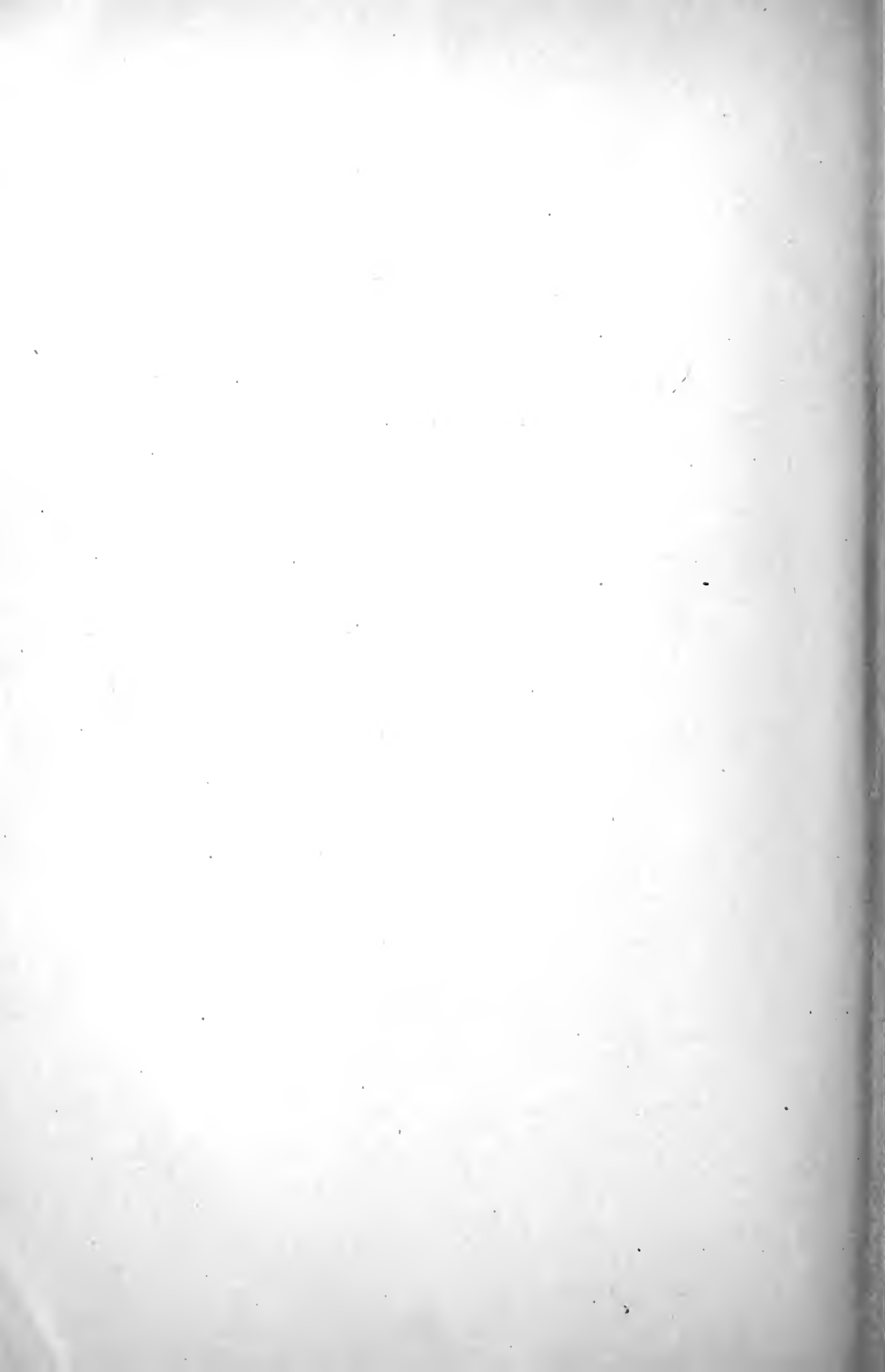


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(Noguchi: *Cristispira* in North American shellfish.)



## THE MULTIPLICATION OF FIBROBLASTS IN VITRO.

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It is well known that the life of tissues cultivated in the plasma of an adult animal lasts only for a certain period of time. 10 years ago, one of the writers attempted to prevent the death of the tissues living under these conditions.<sup>1</sup> It was supposed that if their waste products were removed by frequent washings, the cells would multiply indefinitely. Every few days, fragments of embryonic heart, cultivated in plasma, were washed in Ringer solution, and placed in a fresh medium. The duration of their life was very much increased, but death still occurred sooner or later. No tissue could be kept alive for more than 3 months.<sup>2</sup> It was evident that the adult plasma did not contain the substances necessary for the indefinite multiplication of fibroblasts. This fact was confirmed by Quagliariello,<sup>3</sup> who injected serum proteins into dogs, and found that they are not used as food by the tissue cells. In the more recent experiments of Kerr, Hurwitz, and Whipple,<sup>4</sup> there was no evidence that serum proteins may be considered as intermediary products between food proteins and parenchyma proteins. Although connective tissue cannot live indefinitely in plasma, it often displays a great activity for several days or weeks. What is the origin of the substances used by the cells in the process of multiplication? The opinion of Lewis,<sup>5</sup> and Ingebrigtsen<sup>6</sup> is that the tissues grow within the limits determined by the amount of food stored up in the body of each individual cell. Burrows<sup>7</sup> also believes that the food material of the tissues is not derived from the medium but from the cells within the fragment. The growth observed in the culture would be a simple transfer of material from the cells of the center of the fragment to those which have migrated into the medium. When growth ceases after a few transplanta-

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<sup>1</sup> Carrel, A., *J. Am. Med. Assn.*, 1911, lvii, 1611.

<sup>2</sup> Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

<sup>3</sup> Quagliariello, G., *Arch. fisiol.*, 1912, x, 150.

<sup>4</sup> Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918-19, xlvii, 356.

<sup>5</sup> Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277.

<sup>6</sup> Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421.

<sup>7</sup> Burrows, M. T., *Anat. Rec.*, 1916-17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

tions, the sum of the total growth would be less than the original mass. Although no accurate measurements have been made, there is no doubt that a fragment of tissue cultivated in the plasma of an adult animal does not increase materially in size, in spite of the active proliferation of the cells. But the cause of this temporary activity has not as yet been ascertained.

It is also well known that the addition of embryonic tissue juice to the plasma of an adult animal activates the rate of cell division and brings about an immense increase in the mass of the tissue.<sup>8</sup> A strain of fibroblasts derived from a small fragment of embryonic heart has produced about 30,000 cultures in the past 9 years, and is as active today as at the beginning of its life. If this strain had been allowed to grow freely, the volume of tissue so produced would be very much larger than the earth. There is no doubt, therefore, that when embryonic juice is added to adult plasma new cells are made from the substances contained in the medium, and that this process can go on indefinitely.<sup>9</sup>

It then appears that adult plasma alone, and plasma mixed with embryonic juice, differ widely in their action on the growth of tissue. The reason for these differences is still incompletely known. It is certain that a mixture of embryonic juice and adult plasma had the power to increase the rate of cell division. But the part played respectively by the constituents of the medium in the multiplication of the cells is not thoroughly understood. The purpose of this article is to investigate whence come the substances used by the fibroblasts cultivated in adult plasma alone, and what constituents of the medium are responsible for the increase of the mass of the tissue when embryonic juices are added to the plasma.

#### I.

Although the mass of a tissue cultivated in the plasma of an adult animal does not increase, or increases very little, the building of the new cells requires some material which must come from the plasma or from the tissue itself. In order to determine the part played by the plasma, the action of its constituents, fibrin and serum, was separately studied. Connective tissue having been found to grow as extensively in fibrin fixed by formaldehyde as in normal fibrin, the rôle of fibrin must be considered as purely mechanical. In a first series of experi-

<sup>8</sup> Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273. Carrel, A., *J. Exp. Med.*, 1913, xviii, 287; 1914, xx, 1.

<sup>9</sup> Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.



ments, the influence of serum was investigated by measuring the growth of connective tissue in media containing no serum, and serum of different concentrations. In a second series of experiments, the possible action of substances contained in the tissues themselves was examined.

*1. Rate of Growth of Tissue in Media Containing Varied Dilutions of Serum.*—Fragments of embryonic heart and of a 9 year old strain of fibroblasts were cultivated in media composed of fibrinogen suspension and of Tyrode solution, and containing no serum, or serum in varied dilutions. The serum was taken from the plasma of chickens which had fasted 24 hours. The animals were about 2 years old and in good health. The serum was preserved in paraffined or Pyrex tubes, and its hydrogen ion concentration was measured. Sometimes it was found slightly modified after a few days. The serum was diluted with Tyrode solution sterilized by filtration through a Berkefeld filter. The suspension of fibrinogen was prepared by a technique already known.<sup>10</sup> A medium composed of 10 per cent fibrinogen suspension and 90 per cent Tyrode solution gave a firm coagulum in which embryonic heart tissue could be cultivated without the occurrence of liquefaction. But if a fragment of old strain of fibroblasts was used, liquefaction took place. A little serum had to be added to the medium to prevent this accident. The other media were made of 10 per cent fibrinogen suspension and of mixtures of Tyrode solution and of serum, such that the concentration of serum varied from 2.37 to 90 per cent. The cultures were prepared and measured according to a method previously described.<sup>11</sup> The area of the original fragment was measured immediately after the preparation of the culture, and 48 hours later. The growth was expressed generally by the relative increase of the surface of the tissue; that is, the total area minus the area of the original fragment, divided by the area of the original fragment. The ratio of the relative increase of the experiment to the relative increase of the control permitted the comparison of the growth of cultures belonging to different experiments or groups of experiments. The absolute increase of the tissue was known by the

<sup>10</sup> Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

<sup>11</sup> Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

width of the ring of new tissue around the original fragment, measured with a micrometer.

In ten experiments, embryonic heart tissue was cultivated in media containing from 0 to 90 per cent serum (Table I). The growth of every fragment in the experimental medium was compared to the growth of a control in plasma. The increase of the tissues was expressed by the width of the ring of fibroblasts. The experiments in which the layer of new tissue was of unequal thickness were discarded. The figures of Table I show that the tissues grew at least as well in Tyrode solution containing no serum as in plasma. The growth was larger in media, the serum concentration of which varied from 2.37 to 85 per cent, than in plasma. When the amount of growth obtained in a given concentration of serum was compared with that obtained, not in plasma, but in another concentration of serum, it was found that the action of a medium containing 2.37 per cent serum was as marked as that of a medium containing 90 per cent serum. The growth of embryonic heart appeared to be independent of the concentration of the serum as long as the amount of fibrin was not modified. This shows that the cells in their process of multiplication do not make use of the serum to an extent measurable by the present method.

In thirty experiments, an old strain of fibroblasts was used instead of embryonic heart (Table II). It did not grow in a medium composed only of fibrin and Tyrode solution, because the coagulum liquefied in a few hours. There was practically no difference between the amount of growth of the fragments cultivated in media the serum concentrations of which varied from 2.37 to 80 per cent. When fragments of the old strain were cultivated in 10 per cent serum and 50 per cent serum, and kept in the same media for several passages, no difference in the amount of new tissue was observed, even after four passages, as is shown by Text-fig. 1. In several experiments, two fragments of the old strain were studied comparatively in plasma and in 10 per cent serum. The tissues grew at about the same rate, in spite of the great difference in the composition of the medium. The rate of growth decreased rapidly and in about the same way in both media. Death occurred after the sixth or seventh passage. In Text-fig. 2 is shown a typical experiment. The decrease in the rate of growth is more rapid during the first passages in normal plasma

TABLE I.

*Growth of Embryonic Heart Tissue in Plasma and Varied Dilutions of Serum, Expressed in Terms of Width of Ring.*

Experiment No.		Culture No.	Composition of medium.																				Remarks.
			Control, plasma.	Experiment, serum, 0 per cent.	Control, plasma.	Experiment, serum, 2.37 per cent.	Control, plasma.	Experiment, serum, 8.3 per cent.	Control, plasma.	Experiment, serum, 16.5 per cent.	Control, plasma.	Experiment, serum, 20.5 per cent.	Control, plasma.	Experiment, serum, 22 per cent.	Control, plasma.	Experiment, serum, 30 per cent.	Control, plasma.	Experiment, serum, 33 per cent.	Control, plasma.	Experiment, serum, 50 per cent.	Control, plasma.	Experiment, serum, 85 per cent.	
1	18585		0.2	0.4	0.1	0.2	0.2	0.1	0.05	1.1	0.2	1.0											
2	18656																						
3	18700		0.5	Liquefied.	0.4	1.6	0.3	2.0	0.2	0.3						0.2	2.0	0.1	2.0				
4	18735		0.6	1.0	0.1	1.6	1.0	1.0	0.7	1.6													
5	18795		0.8	1.0	0.8	1.1	0.7	1.3	0.8	1.2													
6	18840		0.5	0.5	0.5	2.0	0.4	1.6	0.7	1.8													
7	18864		1.5	2.0	1.5	4.0	1.6	4.5	1.3	3.3													
8	18916		0.8	2.0	0.6	2.2	0.5	3.6	0.6	1.2													
9	18951									0.7													
10	18962		0.9	2.5	1.2	1.2	1.9	1.7															
Average.			0.6	1.0	0.6	1.7	0.8	1.9	0.6	1.2	0.2	1.0	0.8	1.7	0.8	1.2	0.9	2.0	0.1	2.0	0.9	1.2	

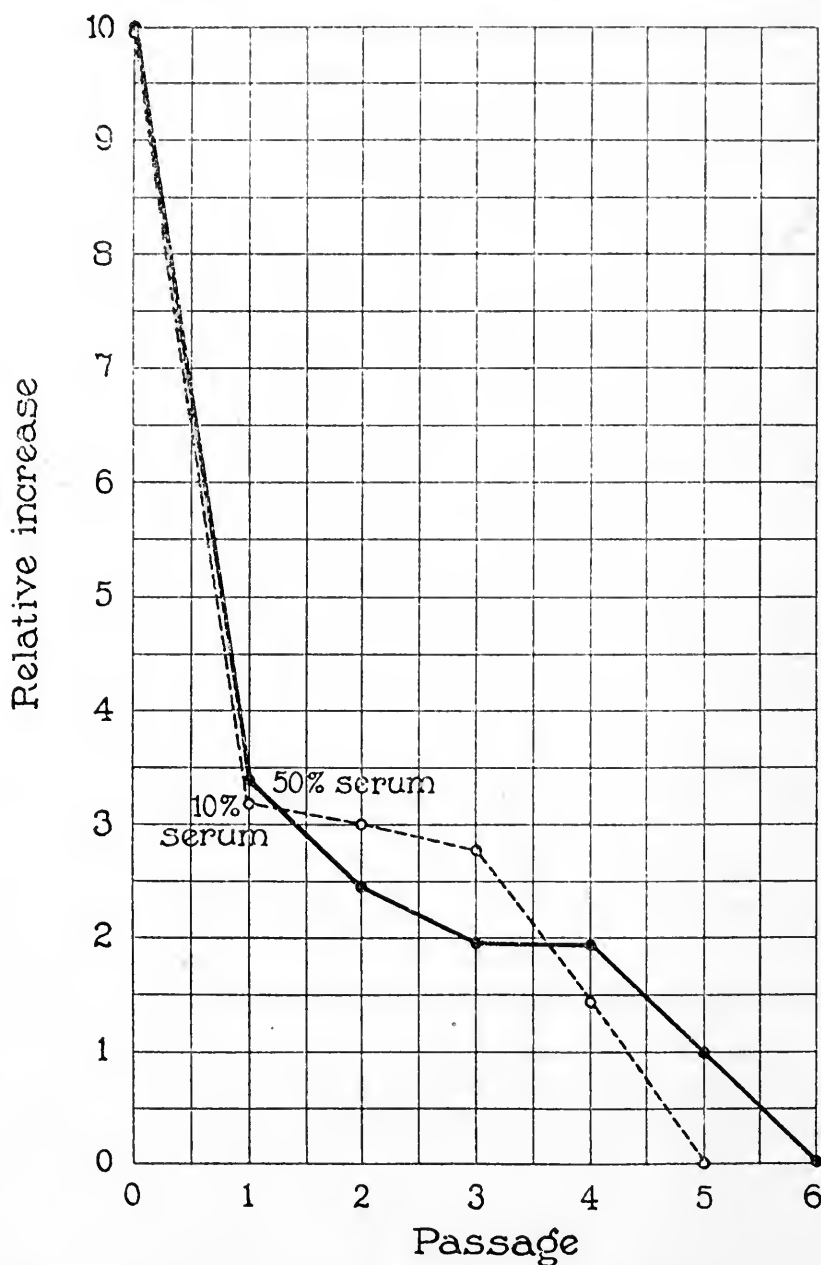
90 per cent serum control instead of plasma.

The increase of the tissues is expressed by the width of the ring of fibroblasts which invaded the medium over a period of 48 hours. In the first nine experiments, the control was cultivated in adult plasma. In the tenth experiment, the control was cultivated in 90 per cent serum instead of plasma.



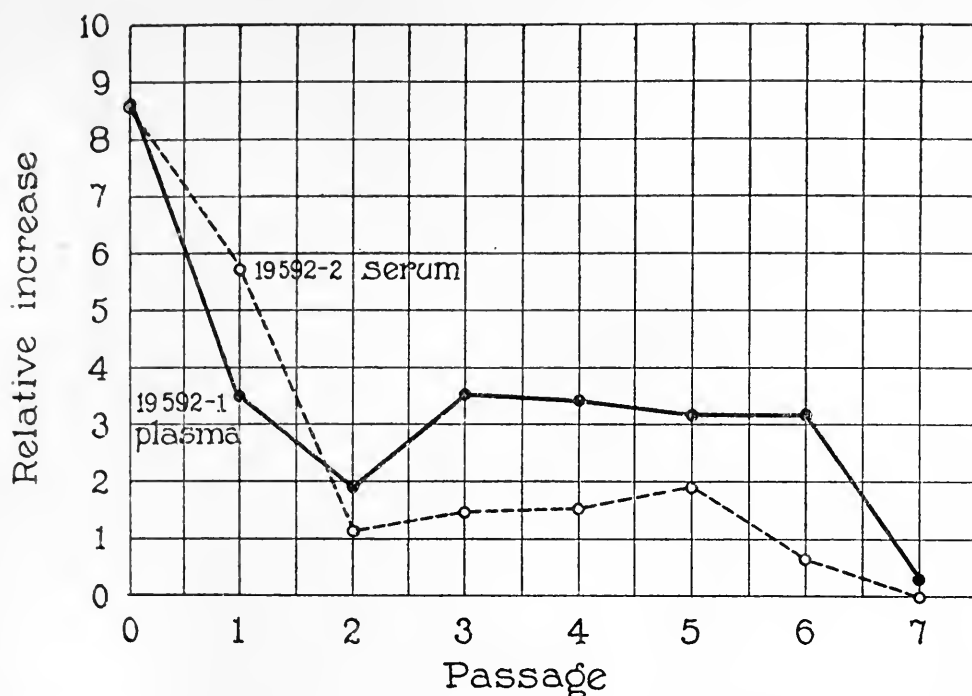


than in a medium containing only 10 per cent serum. Afterwards, there are only small differences in the amount of tissue produced in both media, and death occurs at the same time. This completes the



TEXT-FIG. 1. Comparison of the rate of growth of two fragments of an old strain of fibroblasts in media containing 10 and 50 per cent serum, in the course of five and six passages (Experiment 19364).

demonstration that the fibroblasts in adult plasma or serum do not make use of the serum proteins.



TEXT-FIG. 2. Comparison of the rate of growth of an old strain of fibroblasts in adult plasma and 10 per cent serum, in the course of seven passages (Experiment 19592).

2. *Rate of Growth of Tissue in Plasma.*—Fragments of embryonic heart and of an old strain of fibroblasts were cultivated in adult plasma, transferred into new medium every 48 hours, and studied comparatively. The heart was taken from 10 to 14 day old chick embryos, cut in small fragments in a little Ringer solution, and imbedded in plasma. Coagulation always occurred spontaneously after a few minutes, and the growth was measured at the end of 48 hours. During several passages, the tissues displayed great activity, and the surface of the original fragment increased, as well as the width of the ring of new tissue. The rate of growth reached its maximum after a few passages. Then it progressively decreased and death occurred after from ten to thirty passages. The individual differences in the length of life of the cultures were probably due to the nature of the plasma and to the technique used in the transfer of the tissue. The details of two typical experiments are given as an illustration in Table III and Text-fig. 3. The surface of the original fragment, the surface of the new tissue, and the relative increment of the fragment at

each passage were calculated. In the first experiment, the value of the relative increment increased as far as the fourth passage. At the same time, the area of the fragment became larger and reached its maximum at the fifth passage. Later, it progressively decreased and death occurred after the twelfth passage. In the second experiment there was also an increase in the rate of growth of the fragment and

TABLE III.

*Growth of Embryonic Heart Tissue in Adult Plasma during Fifteen and Eleven Passages (Experiments 18947 and 18948).*

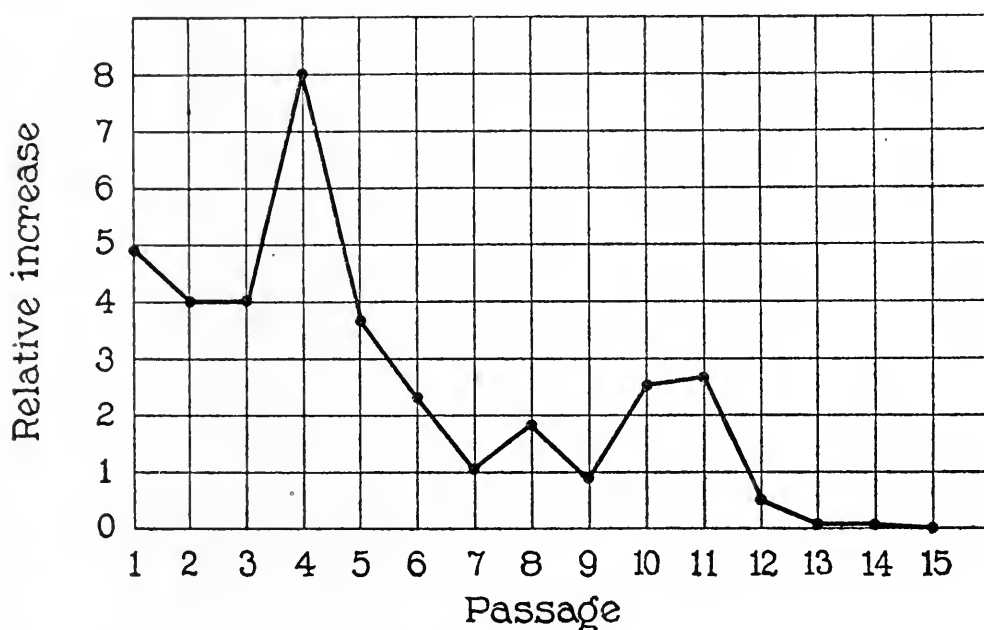
Experiment 1.						Experiment 2.					
Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.	Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
		1920						1920			
1	18947	Dec. 29	1.6	9.1	5.69	1	18948	Dec. 28	1.0	4.9	4.9
2	19006	" 30	1.8	5.7	3.2	2	19007	" 30	1.5	6.0	4.0
		1921						1921			
3	19028	Jan. 1	2.1	12.5	5.92	3	19029	Jan. 1	2.5	10.0	4.0
4	19044	" 3	2.5	19.3	7.7	4	19045	" 3	2.1	16.9	8.0
5	19065	" 5	5.1	20.1	4.0	5	19066	" 5	2.6	9.5	3.65
6	19091	" 7	3.5	11.5	3.85	6	19092	" 7	3.3	7.5	2.29
7	19122	" 10	4.4	4.3	1.0	7	19123	" 10	2.8	3.0	1.07
8	19152	" 12	2.7	2.8	1.0	8	19153	" 12	2.3	4.2	1.8
9	19189	" 14	1.2	0.7	0.06	9	19190	" 14	1.9	1.6	0.89
10	19214	" 15	1.5	5.2	3.45	10	19215	" 15	1.3	3.25	2.5
11	19231	" 17	2.1	Few cells.	0	11	19232	" 17	1.7	4.5	2.65
						12	19255	" 19	1.3	0.7	0.5
						13	19280	" 21	0.7	0.4	0.06
						14	19294	" 22	0.5	0.6	0.1
						15	19314	" 24	0.4	0	0

in their size, the maximum of which was reached at the sixth passage. It was impossible to know whether the active multiplication of the cells and the increase in the area of the original fragment during the first passages meant an increase in the volume of the fragment. This point could be ascertained if the tissues were weighed or the cells counted, but the precision of the method which was used is not sufficient to detect a slight and temporary increase of the mass. It



was obvious, however, that the activity of the cultures reached a maximum after a few passages, then decreased until death occurred. It seemed that in the beginning of its life *in vitro* the growth was activated by something which progressively disappeared.

Fragments of a 9 year old strain of fibroblasts were also grown in adult plasma. Previous to the experiment, the strain was kept in a condition of great activity in a mixture of plasma and embryonic juice. Its relative increase in 48 hours was generally from 6 to 10. The fragments were divided and put into plasma. Coagulation of



TEXT-FIG. 3. Growth of embryonic heart in adult plasma during fifteen passages (Experiment 18948).

the medium was obtained by a small piece of fibrin, because fibroblasts living *in vitro* cannot bring about the clotting of the plasma, as embryonic tissues do. Whenever the tissue was cultivated in adult plasma alone, the rate of growth became very much slower. According to the condition of the adult plasma, the decrease in the rate of growth and in the size of the fragment was more or less rapid. In several cases, death occurred after two or three passages, as shown in Table IV and Text-fig. 4. Before the experiment, the relative increase after 48 hours was 10.5. After the first, second, and third passages in adult plasma alone, the relative increase became respec-

tively 1.3, 0.8, and 0. In other experiments, the life of the old strain in adult plasma was longer, and death occurred after six or ten passages (Text-fig. 2). The variations were due to the degree of activity of the strain previous to the experiment, and to the condition of the plasma.

There was a striking difference between the growth of the fragment of embryonic heart and the strain of fibroblasts. As soon as the strain of fibroblasts was placed in plasma alone, the rate of multiplication of its cells decreased (Text-figs. 2 and 4), while under the same conditions the activity of a fragment of embryonic heart increased for several days (Text-fig. 3). The life of the heart was much longer than that of the strain of fibroblasts. These phenomena could not be

TABLE IV.

*Growth of an Old Strain of Fibroblasts in Adult Plasma during Three Passages (Experiment 18907-2).*

Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
1	{ 18907-2 18947	1920 Dec. 26	5.7	59.3	10.4
		" 28	5.0	6.8	1.36
		1921			
2	19006	Jan. 1	5.3	4.7	0.89
3	19028	" 3	5.3	0	0

attributed to a greater original activity of the embryonic heart, because the rate of proliferation of the fibroblasts of the 9 year old strain is at least as rapid as that of the connective tissue cells of a 10 or 12 day old embryonic heart. They might be due to substances contained in the tissue itself and more abundant in the embryonic tissue than in the strain of fibroblasts. It is known that tissue juices which activate the rate of cell division have also the power of coagulating fluid plasma. Chicken plasma is rapidly transformed into a solid coagulum by a small fragment of embryonic heart, even though the latter has been thoroughly washed in Ringer solution. On the contrary, a fragment of the old strain of fibroblasts does not bring about the coagulation of the plasma. It is, then, possible that embryonic heart contains some of the tissue juice which was found by

one of the writers to be capable of greatly accelerating the rate of cell multiplication,<sup>1</sup> while the strain of fibroblasts lacks it. During the first passages of embryonic heart, the small amount of juice contained in the tissue is probably responsible for the greater activity of the cells. When, after a few days, the original fragment has become surrounded by a dense reticulum of fibroblasts, the rate of growth de-



TEXT-FIG. 4. Growth of an old strain of fibroblasts in adult plasma alone during three passages (Experiment 18907-2).

creases just as it does in a culture of the old strain. It was observed frequently that if a cut were made through the original fragment, after growth had almost ceased, there was a resumption of activity, possibly due to the setting free of some embryonic juice. The activity displayed in adult plasma by both embryonic heart and old strain may be caused by the substances stored within the cells of the tissue, which have the power of increasing the rate of cell division, as is already known.

## II.

The indefinite multiplication of fibroblasts, which occurs as soon as embryonic tissue juice is added to adult plasma, may be explained by two different hypotheses: the embryonic juice renders possible the utilization by the cells of substances contained in the serum or the fibrin; or it supplies itself the material required by the cells for their proliferation.

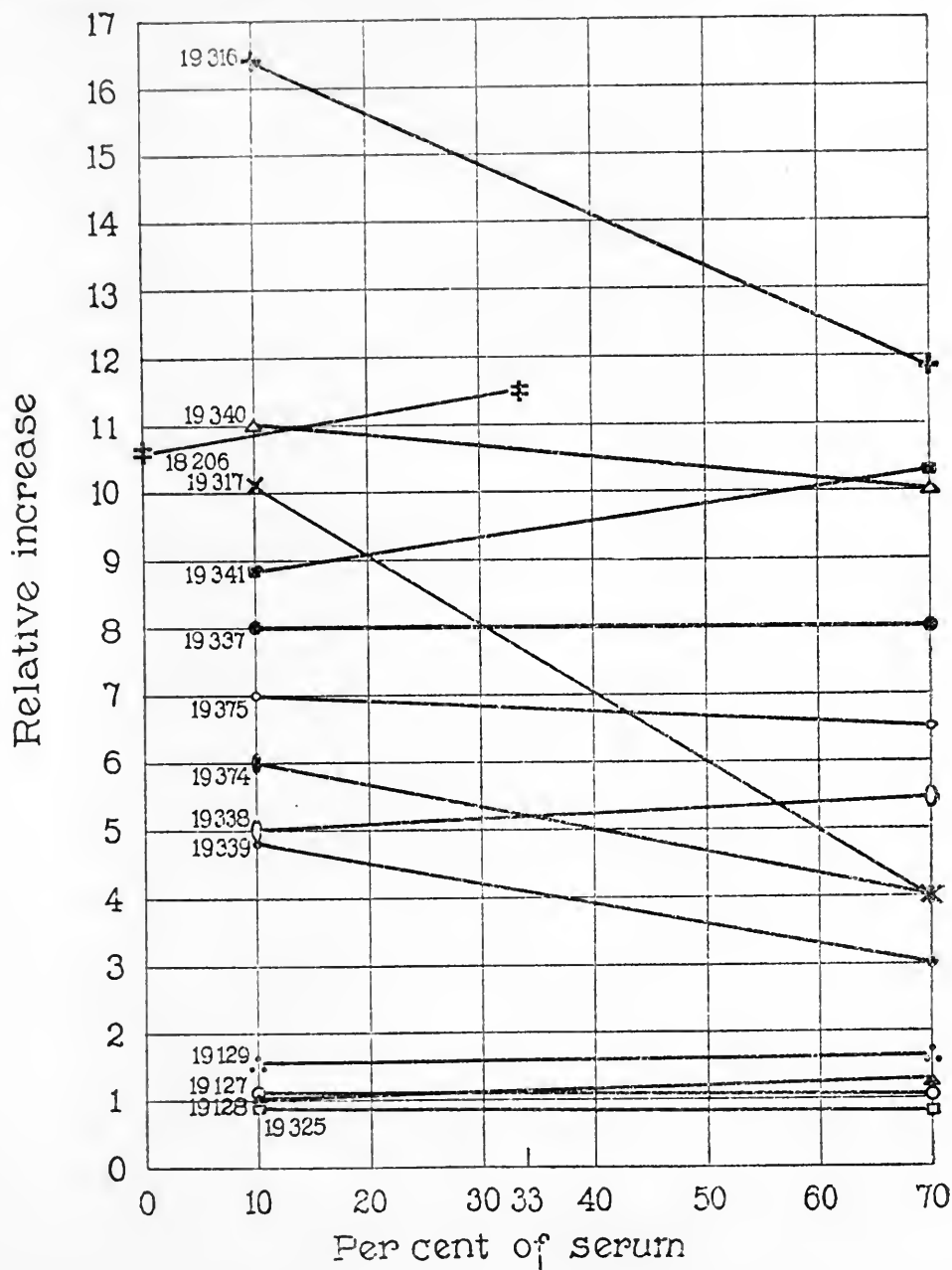
TABLE V.

*Growth of an Old Strain of Fibroblasts in a Medium Composed of a Constant Amount of Tissue Juice and 10 and 70 Per Cent Serum.*

Experiment No.	Culture No.	Date.	Relative increase.			
			Composition of medium.			
			Control.	Experiment.	Control.	Experiment.
			33 per cent.	0 per cent.	10 per cent.	70 per cent.
		1921				
1	18206		11.5	10.75		
2	19316	Jan. 24			16.4	11.8
3	19317	" 24			10.1	4.0
4	19325	" 25			0.9	0.8
5	19337	" 26			8.0	8.0
6	19338	" 26			5.0	5.4
7	19339	" 26			4.8	3.0
8	19340	" 26			11.0	10.0
9	19341	" 26			8.8	10.3
10	19127	" 10			1.1	1.0
11	19128	" 10			1.0	1.2
12	19129	" 10			1.6	1.6
13	19374	" 27			6.0	4.0
14	19375	" 27			7.0	6.5

*Influence of Serum.*—In a first group of experiments, it was attempted to find whether serum was used by the cells in the presence of embryonic juice. Tissue juices were obtained by centrifugation of a pulp made of 10 or 12 day old chick embryos. The media were composed of a constant amount of fibrinogen suspension and embryonic tissue juice, and of varied quantities of serum and Tyrode solution. Fragments of the 9 year old strain of fibroblasts were cultivated in these media. The coagula made of fibrin, embryonic juice, and Tyrode

solution liquefied after a few hours in all the experiments but one. In this experiment, the amount of new tissue was practically as large in



TEXT-FIG. 5. Growth of an old strain of fibroblasts in media containing a constant amount of tissue juice and varied dilutions of serum.

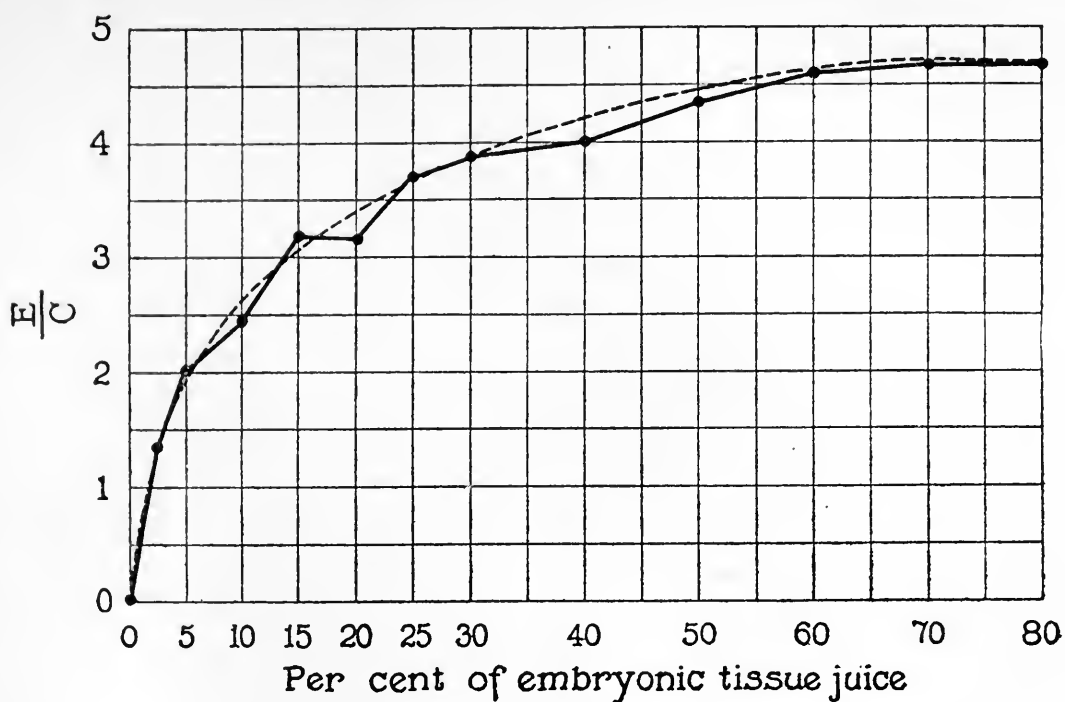
the medium containing no serum as in the medium containing 33 per cent serum. In thirteen experiments the media contained 10

and 70 per cent serum, and their action on the growing tissue was about identical, as is shown by the figures of Table V and by Text-fig. 5. The complete lack of serum, or its presence in low or high concentrations, had no influence on the rate of growth. This fact demonstrated that even in the presence of embryonic juices there was no utilization of the serum by the cells to a measurable extent.

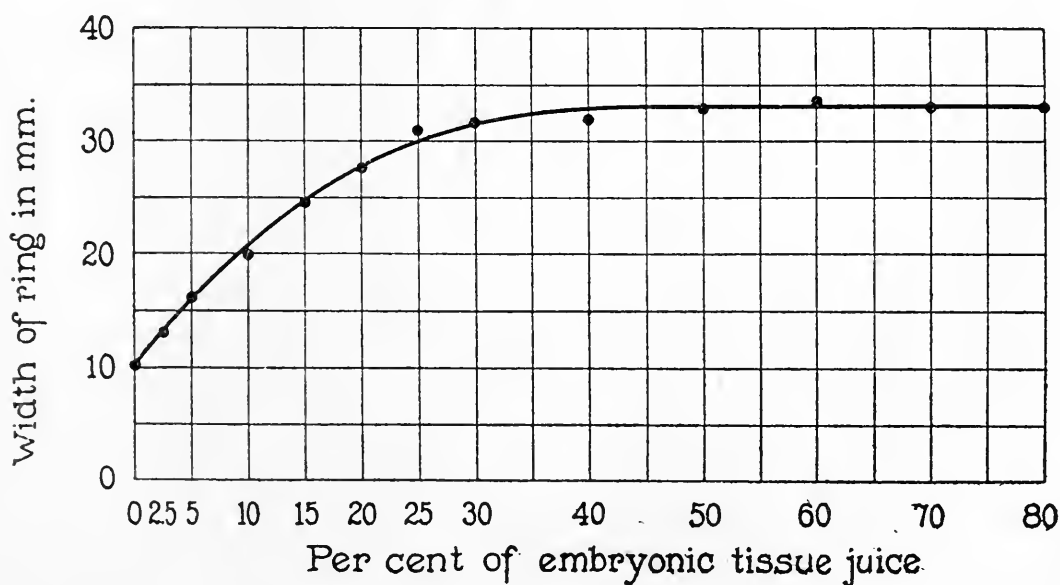
*Influence of Fibrin.*—In a second group of experiments, the action of the fibrin was studied. Plasma was coagulated by a little tissue extract at the surface of a cover-glass, and the coagulum was fixed in 2 per cent formaldehyde solution in Ringer solution for 1 hour. It became slightly bluish, but remained transparent. Then it was washed for 24 hours in distilled water, and for 24 hours in Ringer solution. It was then placed for half an hour in embryonic juice. The excess of embryonic juice was removed with blotting paper, and fragments of embryonic heart and of an old strain of fibroblasts were cultivated in the clot. The growth assumed the same characteristics and extent as in normal fibrin. In 48 hours, the width of the ring of reticulated fibroblasts was from 5 to 6, which is the same as in normal fibrin and embryonic juice. Since the growth in normal fibrin and in fibrin fixed by formaldehyde is the same, it is practically certain that fibrin plays only a mechanical rôle, and is not used by the cells.

*Influence of Embryonic Juice.*—In a third group of experiments, the embryonic juice was added to the medium in progressively increasing concentrations, while the amounts of fibrin and serum were kept constant. The tissues responded by considerable differences in the extent of growth, and these differences were the function of the concentration of the juice.

Fifty-five experiments were made. The action of the medium was ascertained by measurement of both the relative and the absolute increase of the tissue (Table VI). The ratios of relative increases of the experiments and of the control tissues were plotted in ordinates, while the concentrations of the juice present in the medium were plotted in abscissæ. The curve (Text-fig. 6) shows that the cells responded to the addition of small amounts of embryonic juice to the medium by a rapid increase of the rate of multiplication. Increases in concentration from 0 to 10 per cent brought about a greater change in the activity of the cells than increases from 10 to 80 per cent. After



TEXT-FIG. 6. Relation between the ratios  $\left(\frac{E}{C}\right)$  of the relative increases of the tissue in experiments and controls, and the concentration of embryonic juice in the medium.



TEXT-FIG. 7. Relation between absolute increase of the tissue and the concentration of the embryonic juice in the medium.

TABLE VI.

*Relation between the Amount of Growth Expressed in Terms of Relative Increase, Ratios of Relative Increases of Control and Experiments, and of Absolute Increase, and the Concentration of the Embryonic Juice in the Medium.*

2.5 per cent tissue juice.						5 per cent tissue juice.						10 per cent tissue juice.						15 per cent tissue juice.													
Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.		Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.		Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.		Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.									
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.								
19403	4.5	5.4	1.17	9	13	19418	2.85	4.59	1.6	7	15	19437	2.0	5.32	2.66	7	13	19470	2.5	8.92	3.57	13	18								
19404	4.47	6.48	1.44	10	14	19419	3.71	9.46	2.58	7	16	19438	2.0	5.61	2.8	7	19	19471	2.30	9.66	4.2	11	25								
19405	4.7	8.13	1.73	9	13	20043	2.47	5.93	2.41	10	16	20060	2.87	6.81	2.15	11	22	20084	3.02	8.55	2.84	11	27								
20040	2.66	3.83	1.39	10	12	20044	2.21	4.20	1.9	10	18	20061	2.58	5.53	2.14	13	23	20085	4.78	11.21	2.34	12	28								
20041	3.27	4.18	1.27	10	13	20045	2.9	5.71	1.97	10	16	20062	2.58	6.21	2.42	12	22	20086	3.05	10.02	3.28	10	24								
20042	4.61	5.27	1.14	9	13	Average.....					2.09	8.8	16.2	Average.....					2.43	10	19.8	Average.....					3.25	11.4	24.4		
Average.....					1.36	9.5	13	Average.....					2.09	8.8	16.2	Average.....					2.43	10	19.8	Average.....					3.25	11.4	24.4

20 per cent tissue juice.						25 per cent tissue juice.						30 per cent tissue juice.						40 per cent tissue juice.												
Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.		Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.		Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.		Experiment No.	Relative increase.		Ratio, $\frac{C}{E}$	Width of ring.								
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.							
19484	2.34	5.89	2.53	9	17	19505	2.6	9.6	3.7	10	28	19511	3.66	15.0	4.07	10	31	19528	2.3	9.2	4.0	10	30							
19485	3.59	8.52	2.47	12	20	19506	2.83	11.34	4.0	12	27	19512	3.94	15.0	3.8	10	32	19529	2.64	10.3	3.9	9	30							
19509	4.2	16.09	3.81	11	33	19507	3.39	13.3	3.9	11	31	20001	2.33	9.08	3.9	10	33	19530	2.23	9.6	4.3	10	30							
19510	4.18	15.68	3.80	11	35	20087	2.63	8.93	3.4	12	35	20002	3.5	11.79	4.17	9	32	19883	2.74	11.67	4.26	9	35							
19998	3.0	10.97	3.65	12	31	20088	2.56	9.18	3.57	10	33	20003	2.9	10.0	3.45	14	30	19884	3.54	12.71	3.6	10	34							
19999	3.17	9.52	3.02	11	27	Average.....					3.7	11	30.8	Average.....					3.88	10.6	31.6	Average.....					4.01	9.6	31.8	
20000	2.43	7.08	2.9	11	30	Average.....					3.7	11	30.8	Average.....					3.88	10.6	31.6	Average.....					4.01	9.6	31.8	
Average.....					3.17	11	Average.....					3.7	11	30.8	Average.....					3.88	10.6	31.6	Average.....					4.01	9.6	31.8



50 per cent tissue juice.						60 per cent tissue juice.						70 per cent tissue juice.						80 per cent tissue juice.								
Experi- ment No.	Relative increase.		Ratio, $C_1$ / $C_2$	Width of ring.		Experi- ment No.	Relative increase.		Ratio, $C_1$ / $C_2$	Width of ring.		Experi- ment No.	Relative increase.		Ratio, $C_1$ / $C_2$	Width of ring.		Experi- ment No.	Relative increase.		Ratio, $C_1$ / $C_2$	Width of ring.				
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.	Control.	Experiment.	
19531	2.36	9.20	3.9	10	26	19544	2.52	11.6	4.6	12	33	19546	2.47	11.36	4.6	9	33	19620	2.33	10.62	4.58	13	33			
19533	2.74	13.2	4.8	10	31	19545	2.85	13.3	4.66	7	34	19547	2.82	13.4	4.75	10	33	19621	1.93	9.08	4.7	12	33			
19886	2.17	9.77	4.5	14	36	Average.....					9.5	33.5	Average.....					Average.....					4.64	12.5	33	
19885	2.49	9.95	4.0	13	34	Average.....					4.6	9.5	Average.....					Average.....					4.64	12.5	33	
20150	2.76	12.91	4.67	10	35	Average.....					4.6	9.5	Average.....					Average.....					4.64	12.5	33	
20151	2.10	8.74	4.16	11	34	Average.....					4.6	9.5	Average.....					Average.....					4.64	12.5	33	
Average.....					4.37	11.2	Average.....					4.37	11.2	Average.....					Average.....					4.37	11.2	32.7

the concentration of the juice in the medium had reached 40 per cent, no further increase was observed when larger amounts were added. The results were checked by measurement of the width of the ring of the new tissue; that is, by comparing the absolute instead of the relative increases (Table VI). The arithmetic mean of the width of the ring of new tissue of several experiments, made with each dilution of juice, was plotted in ordinates, and the concentration of the juice in abscissæ (Text-fig. 7). The curve (Text-fig. 6) showed the same rapid increase of the rate of growth when a small amount of juice was added to the medium. There was no doubt that the substances used by the fibroblasts came from the embryonic juice and that the activity of the fibroblasts was a function of the concentration of the embryonic juice in the medium.

### III.

#### SUMMARY.

The results of the investigation of the cause of the multiplication of fibroblasts *in vitro* may be summarized as follows: Although the life of fibroblasts in the plasma of an adult chicken which has fasted for 24 hours is not permanent, their proliferation is very active for some time. Are the substances used by the cells in their multiplication supplied by the plasma or by the tissue itself? In media composed of a constant amount of fibrin and of a mixture of Tyrode solution and serum in varied concentrations, the amount of growth appeared to be independent of the concentration, and even of the presence of serum. Serum was evidently not used by the cells. It was also found that fibrin is not utilized. This fact explains the results of the experiments of Lewis, who showed long ago that embryonic tissue can grow extensively in Locke solution. Then, the material from which the new cells are built must come from the tissue itself, as was previously supposed by Lewis, Ingebrigtsen, and Burrows. A comparative study of the growth in adult plasma of embryonic heart and of a 9 year old strain of fibroblasts led to the hypothesis that traces of embryonic juice stored in the original fragments are responsible for the activity manifested by the tissues during their temporary life.

When embryonic juice is added to the plasma of an adult chicken, the rate of multiplication of the fibroblasts increases and their life *in vitro* becomes permanent. Does the presence of embryonic juice determine the use by the cells of substances contained in adult plasma? The tissues were cultivated in media containing a constant amount of fibrin and embryonic juice, and varied concentrations of serum. The rate of growth was found to be independent of the amount of serum contained in the medium. It was also observed that the rate of growth in fibrin fixed in formaldehyde solution did not differ from that in normal fibrin. This fact demonstrated that embryonic juice does not give to the cells the power of using the constituents of plasma. When fragments of the 9 year old strain of fibroblasts were cultivated in media containing a constant amount of serum and fibrin, and varied concentrations of embryo juice, the rate of growth was found to be a function of the concentration of the embryonic juice in the medium. It was, therefore, evident that the material employed by the fibroblasts in their indefinite multiplication *in vitro* was supplied by the embryonic juice.

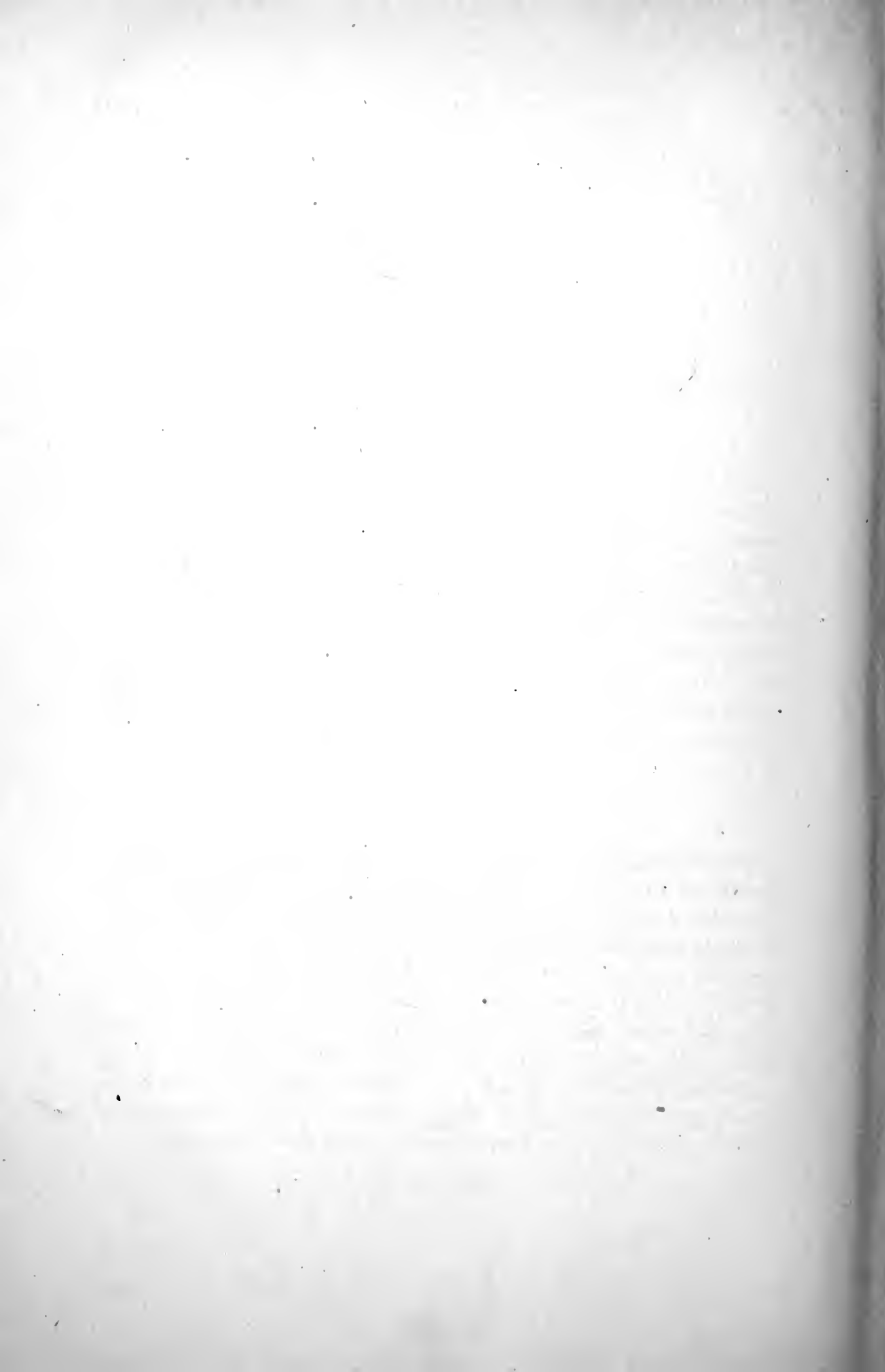
#### IV.

#### CONCLUSIONS.

1. It may be concluded that, under the conditions of the experiments and within the limits of accuracy of the method, the temporary multiplication of fibroblasts cultivated in the plasma of an adult animal is not due to the serum. It may be attributed to the presence of a small amount of embryonic juice within the tissue itself.

2. The indefinite multiplication of fibroblasts in a medium composed of adult plasma and of embryonic juice is due neither to the serum nor to the fibrin. It depends entirely on substances contained in the embryonic juice.

3. There is a definite relation between the rate of growth and the concentration of the embryonic juice in the medium.



## CICATRIZATION OF WOUNDS.

### XI. LATENT PERIOD.

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The latent or quiescent period of cicatrization<sup>1</sup> extends from the time of traumatism to the beginning of contraction. During this stage of apparent inactivity, the mechanism which will bring about the reintegration of the tissues is progressively set in motion. A study of the latent period may lead to a better understanding of the secondary causes which directly or indirectly derive from the injury acting as primary cause, and are instrumental in starting regeneration. The purpose of this article is to examine the duration of the latent period, its transition to the period of contraction, and the characteristics of the curve expressing it.

#### I.

##### *Method.*

The experiments were made with wounds of geometric shape, obtained by excision of a flap of skin in the dorsal region of dogs. The animals were of medium size, short haired, and of quiet temper. 24 hours previous to the operation, they were given a warm bath after the hair had been clipped. Later on the same day, the skin of the dorsal region was washed a second time with soap and water. After the animal had been etherized, the skin was shaved, carefully washed with soap and warm water, and covered for 10 minutes with compresses soaked in 75 per cent alcohol. Then the animal was placed on the operating table and the skin was painted with tincture of 10 per cent iodine, which was allowed to dry for 10 minutes.

<sup>1</sup> Carrel, A., *J. Am. Med. Assn.*, 1910, lv, 2148.

The wounds were generally obtained by resection of a rectangular flap of skin. In some cases, circular flaps were extirpated by means of a sharp edged tube, 2 cc. in diameter. Hemostasis was secured by compression with gauze pads, or by temporary clamping of the small vessels. No ligatures were used, because after a few days they become centers of colonization for the bacteria existing on the surrounding skin. The measurement of the wounds was made in two different ways. In the earlier experiments, the width of a rectangular wound was ascertained with a compass and its variations were studied, instead of the change in the total area. In the more recent experiments, the area of the wound was measured, according to a technique previously described,<sup>2</sup> and expressed in square centimeters. An accurate appreciation of the area required a great deal of care. It is well known that the skin of the dog is not adherent to the aponeurosis, and is very mobile. This causes the size of the wound to become modified by slight changes in the position of the animal. Therefore, in spite of the precautions which were taken, variations occurred in the area of the wounds which were due only to experimental errors. At the end of the latent period, when the edges of the wound had become fixed to the bottom by granulation tissue, the method was much more accurate.

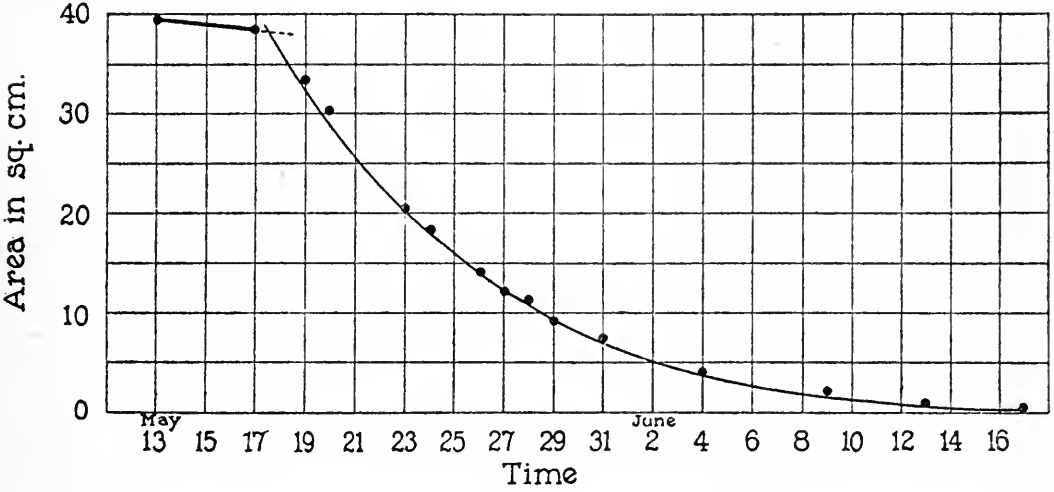
As the purpose of the experiments was to study the latent period under ordinary conditions, the dressing consisted only of talcum powder, paraffin, or plain gauze. The bacterial condition of the surface was ascertained merely by examination of films. Often the wound could be maintained in a condition of surgical asepsis, that is of mild infection, when the dressing was properly fixed to the skin. Infection was usually carried from the surrounding skin to the wound by the gauze, which moves about on the surface of the skin due to the constant movements of the animal. This could be prevented by stitching a gauze pad to the skin itself. Then a thick cotton pad was applied, fixed by bandages, and protected by a shirt.

Seven experiments are briefly described, and the appearance of the latent period is shown by Text-figs. 1 to 7. The width of the wound in centimeters, or its area in square centimeters, is plotted in ordinates and the time in abscissæ. The latent period is designated

<sup>2</sup> Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 430.

by the heavy line. The light line expresses the area of the wounds calculated by the formula of du Noüy,<sup>3</sup> which, of course, does not apply to the widths. In Text-figs. 5 to 7 the curves are arbitrary. The observed area, or width, is represented by the heavy points. In the first experiment, the latent period and the two subsequent periods are described, and the curve expresses the complete phenomenon from the excision of the flap to the last stage of epidermization. In the six other experiments, the latent period and the beginning of the contraction period only are shown.

EXPERIMENTS.



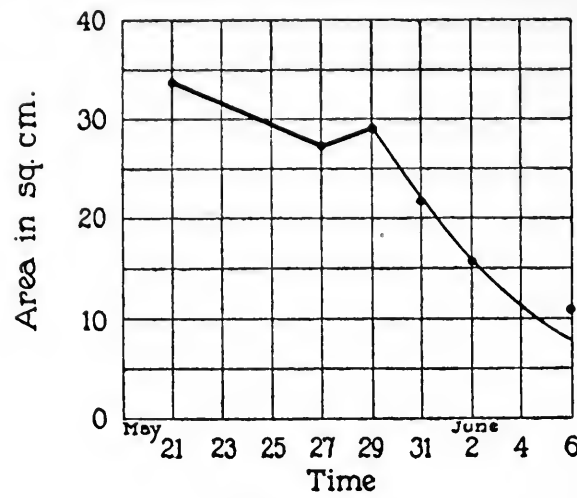
TEXT-FIG. 1.

*Experiment 1. Latent Period and Periods of Contraction and Epidermization.*—Very old female dog, about 13 years of age, No. 1. May 13, 1919. Resection of a rectangular flap of skin on dorsal region. Dressing with dry gauze. Subsequent dressings, dry gauze after washing with neutral sodium oleate. Dakin's solution compress when bacteria appeared in the films.

Date.....	May 13	17	19	20	23	24	26	27	28	29	31	June 4	9	13	17
Observed area...	39.4	38.7	33.5	30.2	20.3	18.7	14.3	12.2	11.5	9.2	7.7	4.0	2.7	1.1	0.5
Calculated area...					20.0	18.3	14.2	12.2	11.0	9.2	7.1	3.8	1.6	0.8	0.3

For the calculated curve, the index *i* was equal to 0.058.

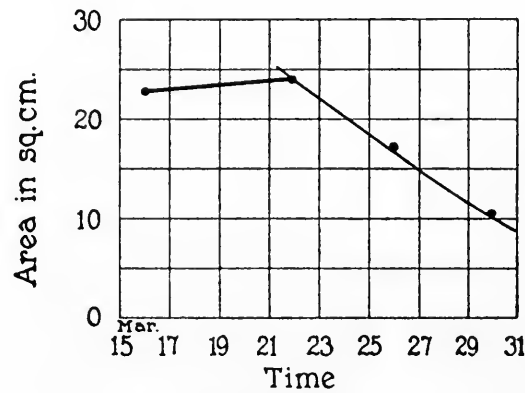
<sup>3</sup> du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451.



TEXT-FIG. 2.

*Experiment 2. Latent Period and Abrupt Beginning of Contraction.*—Female dog, about 2 years old, No. 2. May 21, 1919. Excision of a rectangular flap of skin. Dry gauze dressing. Washing with sodium oleate in subsequent dressings.

Date.....	May 21	27	29	31	June 2	6
Observed area. ....	33.7	27.4	29.0	21.7	15.8	11.0

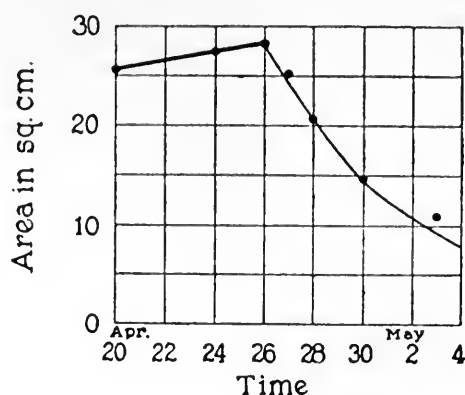


TEXT-FIG. 3.

*Experiment 3. Latent Period and Period of Contraction.*—Male dog, about 2 years old, No. 3. March 16, 1920. Excision of a rectangular flap of skin. Dry gauze dressings.

Date.....	Mar. 16	22	26	30
Observed area. ....	23.0	24.0	17.3	10.6

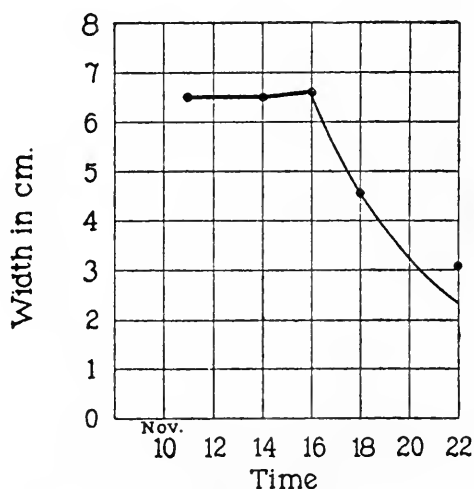




TEXT-FIG. 4.

*Experiment 4. Latent Period and Period of Contraction.*—Male dog, about 1 year old, No. 4. April 20, 1920. Excision of flap of skin. Dry gauze dressing. Washing with neutral sodium oleate in subsequent dressings.

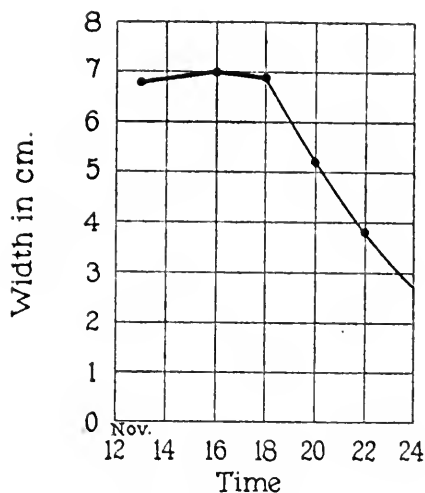
Date.....	Apr. 20	24	26	27	28	30	May 3
Observed area.....	25.8	27.4	28.4	25.1	20.7	14.7	11.0



TEXT-FIG. 5.

*Experiment 5. Latent Period and Abrupt Beginning of Contraction.*—Young male dog, about 11 months old, No. 5. November 11, 1907. Resection of a rectangular flap of skin in dorsal region. Measurement of the width of the wound with a compass. Talcum powder dressing. November 16. Beginning of granulations. Subsequent dressings, dry gauze.

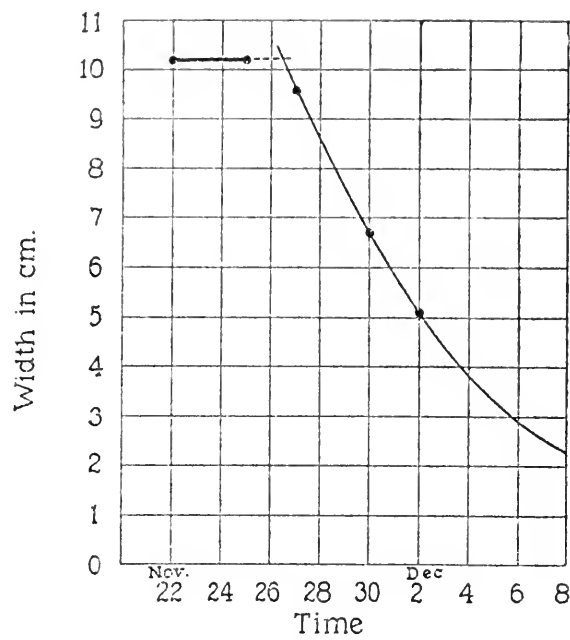
Date.....	Nov. 11	14	16	18	22
Width of wound.....	11.0	6.5	6.6	4.6	3.1



TEXT-FIG. 6.

*Experiment 6. Latent Period and Abrupt Beginning of Contraction.*—Young male dog, about 13 months old, No. 6. November 13, 1907. Resection of a rectangular flap of skin. Measurement of the width of the wound with a compass. Talcum powder dressing. November 18. Small irregular granulations.

Date.....	Nov. 13	16	18	20	22
Width of wound.....	6.8	7.0	6.9	5.2	3.8



TEXT-FIG. 7.

*Experiment 7. Latent Period and Period of Contraction.*—Young male dog, about 10 months old, No. 7. November 22, 1907. Resection of a rectangular flap of skin. Measurement of wound with a compass. Talcum powder dressing. November 27. Granulations began.

Date.....	Nov. 22	25	27	30	Dec. 2
Width of wound.....	11.0	10.2	9.6	6.7	5.1

## II.

## DISCUSSION.

The duration of the latent period varied from 5 to 7 days. It was found, in the course of many other experiments, that the limits of variations may be wider. While the subsequent stages of cicatrization are not affected by external factors, it appears that the latent period may be easily modified in its duration by many local causes, such as mechanical irritation of the tissues, infection, and even by the diet of the animal, as was shown by Clark.<sup>4</sup> The experiments described above deal only with the latent period under ordinary conditions of mild bacterial and mechanical irritation, which was generally obtained by dry gauze dressings. During the first stage of healing, the area was generally found to remain constant. However, in Experiment 7 an increase of the surface, and in Experiment 5 a decrease followed by an increase of the surface, were observed. These changes were probably due to errors of observation. As long as the edges of the wound were not fixed to the bottom by granulating tissue, a slight modification of the tension of the surrounding skin could modify the area considerably. The appearance of the granulation tissue generally announced the end of the latent period. But there was no definite relation between both phenomena. The end of the latent period was marked not only by the growth of granulation tissue, but by an abrupt beginning of the contraction period, which immediately acquired its maximum velocity. In Experiments 4 and 7, there was a transition period which lasted possibly 24 hours, and during which the contraction started slowly. As a rule, the onset of contraction was sudden and a period of maximum activity succeeded the period of complete quiescence.

The formula of du Noüy was found to apply accurately to the beginning of the period of contraction. On the graphs, it may be seen that the light line representing the calculated area follows the observed area almost constantly. There was a perfect coincidence between the calculated and the observed surfaces in Experiment 1, even during the first hours of the contraction. An examination of the seven graphs shows that the phenomenon could not be accurately

<sup>4</sup> Clark, A. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 117.

expressed by a curve presenting a point of inflexion. So far, this fact has not been emphasized, and, on account of an insufficient description of the conditions of the experiments, certain curves, in previous publications, were misleading.<sup>5</sup> Recently, Fauré-Frémiet and Vlès<sup>6</sup> attempted to apply to the cicatrization process the equation expressing the monomolecular autocatalytic reaction used by Robertson<sup>7</sup> in his studies on growth. As a consequence of this hypothesis, the curve of the areas must pass by a point of inflexion, in conformity with the equation

$$\frac{S}{S_0 - S} = e^{KS_0 (T - t_{\frac{1}{2}})}$$

which necessitates that the rate should be maximum at the time  $t_{\frac{1}{2}}$  where  $S = \frac{S_0}{2}$ . One of the curves calculated in this manner agrees fairly closely with that of du Noüy. The other presents a point of inflexion; that is, a maximum in the rate of healing during the period of contraction. There is evidently a close relation between this curve and the observed curve of an experiment previously reported.<sup>5</sup> But the point of inflexion of this observed curve was due to a mere accident. The wound was old and infected, and had begun to cicatrize under a plaster of Paris splint. The observed curve showed the variations of the rate of cicatrization both before and after normal cicatrization had really begun. As these details were not mentioned in the paper, Fauré-Frémiet and Vlès<sup>6</sup> could not help being misled by this accidental fact. Although their formula and the formulas published by de Beaujeu<sup>8</sup> and Lumière<sup>9</sup> show a lack of agreement with the observed facts, there is no doubt that the curve representing the normal process of cicatrization may be expressed by more than an equation, as has already been pointed out by du Noüy.<sup>10</sup>

<sup>5</sup> Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 432.

<sup>6</sup> Fauré-Frémiet, E., and Vlès, F., *Compt. rend. Acad.*, clxviii, 363.

<sup>7</sup> Robertson, T. B., *Principles of biochemistry*, Philadelphia, 1920, 475.

<sup>8</sup> de Beaujeu, J., *J. Exp. Med.*, 1917, xxvi, 81.

<sup>9</sup> Lumière, A., *Bull. Acad. méd.*, 1918, lxxix, series 3, 213; *Rev. chir.*, 1917, liii, 656.

<sup>10</sup> du Noüy, P. L., *Recherches expérimentales et application des méthodes de mesure et de calcul à un phénomène biologique: la cicatrization*, Thèse de Paris, Paris, 1917.

But these equations must apply as well to the beginning of the contraction period as to the end of the epidermization period. A glance at the figures and at the chart expressing the observed and calculated areas of Experiment 1 shows that the coincidence is almost perfect and quite satisfactory, in view of the percentage error necessarily involved in experimental wounds on dogs.

Several equations have already been applied by du Noüy to the curve of cicatrization. The first<sup>11</sup> was expressed as follows:

$$T = K_1 \operatorname{Log}_e \frac{S_o}{S} + 2K_2 (\sqrt{S_o} - \sqrt{S})$$

The second<sup>12</sup> has proven more satisfactory:

$$S = S_o e^{-K(T + \frac{T^2}{2p})}$$

The figures calculated by this last equation coincide almost exactly with those calculated by means of the well known extrapolation formula:<sup>3</sup>

$$S_n = S_{n-1} [1 - i(t + \sqrt{t + nt})]$$

They applied to the first stage of contraction as accurately as to the later periods of cicatrization.

### III.

#### CONCLUSIONS.

1. The latent period of cicatrization varies generally from 5 to 7 days.
2. It stops abruptly and contraction starts with its maximum velocity.
3. The formula of du Noüy applies to the beginning of the contraction period as well as to the subsequent periods.

<sup>11</sup> du Noüy, P. L., *J. Exp. Med.*, 1917, xxv, 722.

<sup>12</sup> du Noüy, P. L., *J. Exp. Med.*, 1919, xxix, 329, Text-fig. 4.

## THE DIASTATIC ACTIVITY OF THE BLOOD IN EXPERIMENTAL HYPERGLYCEMIA.

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### I.

#### INTRODUCTION.

Since the demonstration of diastatic activity of the blood and lymph by Magendie, Bial, and Röhman, the production of hyperglycemia and glycosuria by Claude Bernard, and the demonstration of the influence of the pancreas upon carbohydrate metabolism by von Mering and Minkowski, numerous investigators have conducted further studies. It has long been known that the injection of such substances as adrenalin, strychnine, morphine, and more recently, saliva, pituitrin, and various other glandular extracts possessing amylolytic properties leads in some instances to the development of hyperglycemia and glycosuria. In spite of considerable conflict concerning the diastatic content of the blood in these conditions the mass of evidence so far accumulated supports the belief that little or no change is produced. However, Watanabe found in rabbits that the intravenous injection of human saliva and the intraperitoneal administration of sodium bicarbonate (5 or 10 gm.) each produced an increase, while the parenteral administration of soluble starch and the intravenous injection of sodium bicarbonate (5 or 10 gm.) each produced a slight increase in the diastatic activity of the blood.

Further observations have been made concerning the rôle of the pancreas. Following ligation of the duct of Wirsung, Schlesinger reported an increase in the diastatic activity of the blood; Clerc and Loeper verified this result in rabbits; Gould and Carlson, working with dogs, ligated both pancreatic ducts and found a marked increase in the diastatic power of the serum, which they assumed to be due to absorbed amylopsin; King observed an increased diastatic activity in the serum and urine upon ligation of both pancreatic ducts. Wohlgemuth, Noguchi, and King reported a quantitative increase in the diastatic activity of the urine following ligation of both pancreatic ducts.

Bainbridge and Beddard, as well as Carlson and Luckhardt, reported that in cats the concentration of blood diastase is not materially changed by excision of the pancreas; Schlesinger states that pancreatectomy leads to complete disappearance of amylase from the blood. Otten and Galloway found that in dogs immediately following pancreatectomy the concentration of blood diastase sinks rapidly, then rises slightly, remaining at a constant level for a time but never returning to the normal level again. King reports a reduction in the amount of blood diastase in depancreatized dogs. Milne and Peters obtained a decided increase in the blood diastase after complete pancreatectomy.

Recently, the question of the diastatic activity of the blood has been more accurately studied in human diabetes. Myers and Killian (1917) reported an increase in the diastatic activity of the blood in cases of human diabetes. Still more recently (November, 1920), Lewis and Mason, employing essentially the same method as Myers and Killian, found a distinct decrease in the blood diastase in human diabetes.

## II.

### *Method.*

The methods for the determination of the diastatic activity of the blood have varied from time to time but all depend upon a hydrolytic process. Wohlgemuth (1908) based his method upon the hydrolysis of starch into erythrodextrin by amylase, using the simple iodine reaction. Myers and Killian (1917) devised a method based upon the hydrolysis of starch into glucose, using Myers and Bailey's modification of the Lewis-Benedict method for the blood sugar determination.

In our laboratory the method used was essentially the same as that described by Myers and Killian with the exception of the substitution of Folin and Wu's method for blood sugar determination.

Our experiments consisted (1) in observing the effect upon blood diastase in rabbits after the subcutaneous injection of phlorhizin, uranyl nitrate, morphine sulfate, and adrenalin; (2) in noting the effect of puncture of the floor of the fourth ventricle; and (3) in observing the effect upon the content of blood diastase in completely depancreatized dogs. The controls consisted in noting (1) the effect of diet; (2) the effect produced by repeated bleeding of the animal comparable to the procedure followed in the experiment; (3) the effect of asphyxia; (4) the effect of ether anesthesia; and (5) the effect of a surgical operation similar in extent to pancreatectomy.<sup>1</sup>

<sup>1</sup> All operations were performed under ether anesthesia.



The rabbits were fed upon a diet consisting of carrots, oats, and water; the diet for the dogs was composed of a balanced mixture of beef heart, casein, crackers, salt, lard, and water. The specimens of blood were taken from the ear veins of the rabbits and by cardiac puncture from the dogs. All blood diastase determinations were started within 10 minutes after withdrawal of the blood. The examinations in the case of the rabbits were made 2 hours following the injection and the puncture; and then at intervals of 24 hours until the blood sugar had returned to normal or the animal died. After pancreatectomy in dogs the examinations were made immediately following the operation in two cases; in all animals 2 hours later, and then at 12 hour intervals until the animal succumbed.

All drugs were given subcutaneously in the following doses: morphine sulfate, 2 cc. of a 4 per cent solution (80 mg.); adrenalin chloride, 2 cc. of 1:1,000 solution; uranyl nitrate, 3 mg.; phlorhizin, 0.25 gm. The Eckhard operation was performed in two of the successful puncture experiments. The pancreatectomies consisted in complete removal of the pancreas by usual operative technique under ether anesthesia without morphine.

### III.

#### EXPERIMENTS.

Three rabbits were given phlorhizin subcutaneously in doses of 0.25 gm. dissolved in warm distilled water. In one animal with a normal diastatic activity ranging between 3 and 8 mg. per 100 cc. the first injection of phlorhizin produced no change in diastatic activity, but a second injection 5 days later was followed in 24 hours by a figure for diastase of 20 mg. At this time, however, that animal had been bled ten times in 28 days and the result was probably in part influenced by the repeated bleedings, and therefore can only be regarded as indicating a tendency to increase. In another animal with normal figures of 2 to 5 mg. the first injection of phlorhizin was followed by a rise to 11 mg., again a tendency to increase, but a second injection in the same animal showed no change. A third animal showed no change following two separate injections. Table I is the protocol of one animal.

TABLE I.

*Effect of the Subcutaneous Injection of Phlorhizin. Rabbit 1.*  
Weight 1.2 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Dias-tase* per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	a.m.	mg.	mg.			
July 22-Aug. 7..		{ 112 139	3 8	Negative.	Negative.	Normal amounts with minimal and maximal of six determinations. Phlorhizin, 0.25 gm. subcutaneously.
Aug. 12.....	11.30					
	p.m.					
	1.30	122	4			
" 13.....	1.30	131	9	Negative.	Negative.	Phlorhizin, 0.25 gm. subcutaneously. High diastase probably due to repeated bleedings.
" 14.....	1.30	128	3	Positive.	"	
	a.m.					
" 20.....	11.30					
	p.m.					
	1.30	137	20	Positive.	Negative.	
" 21.....	1.30	128	9	"	"	
" 22.....	1.30	137	14	"	"	
	a.m.					
" 30.....	11.30					Phlorhizin, 0.25 gm. subcutaneously.
	p.m.					
	1.30	122	9	Positive.	Positive.	
" 31.....	1.30	115	2	"	"	

\* Throughout the tables "diastase" refers to the amount of sugar produced by the action of the blood upon soluble starch.

Four rabbits were given uranyl nitrate in doses of 0.003 gm. of pure salt. All four showed albuminuria and glycosuria, and all were fatally intoxicated and showed acute nephritis at autopsy. None of these showed hyperglycemia and none showed any increase in diastatic activity of the blood even when moribund. Table II is the protocol of one animal.

Five rabbits were given doses of 0.080 gm. of morphine sulfate subcutaneously. Collectively fourteen injections of morphine were given. All showed some degree of hyperglycemia, three showed

glycosuria, and all showed respiratory disturbance. A definite increase in diastase occurred when severe hyperglycemia was produced. Table III is a protocol of one animal.

Six rabbits were given adrenalin (Parke, Davis and Company) in doses of 2 cc. of 1:1,000 solution from a freshly opened bottle. Collectively eight injections were given. All showed hyperglycemia

TABLE II.

*Effect of the Subcutaneous Injection of Uranyl Nitrate. Rabbit 2.*  
Weight 1.1 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	a.m.	mg.	mg.			
Aug. 25–Sept. 3.		{ 110 192	{ 4 10	Negative.	Negative.	Normal amounts with maximal and minimal of six determinations. Uranyl nitrate, 3 mg. subcutaneously.
Sept. 17.....	11.00					
	p.m.					
	1.00	128	4	Negative.	Negative.	
" 18.....	1.00	125	4	"	"	
" 19.....	1.00	156	4	"	"	
" 20.....	1.00	172	6	Positive.	"	
" 21.....				"	"	
" 22.....	1.00	185	8	"	0.3percent.	
" 23.....				"	Negative.	
" 24.....	1.00	140	7	"	"	
" 25.....				"	"	
" 26.....						Died. Acute tubular nephritis.

and glycosuria. In one animal with control figures ranging between 4 and 6 mg. per 100 cc. the first injection of adrenalin was followed by a figure of 11 mg. for blood diastatic activity. All others showed no increase. Table IV is a protocol of one animal.

Puncture of the floor of the fourth ventricle was performed in three animals, one by the Claude Bernard method and two by the Eckhard method. All showed hyperglycemia, two showed gly-

cosuria, and none showed any definite increase in diastatic activity. Table V is the protocol of one animal.

Seven rabbits were placed on diets alternately of green vegetables and of carrots, oats, and water. There was no alteration

TABLE III.

*Effect of the Subcutaneous Injection of Morphine Sulfate. Rabbit 3.*  
Weight 1.6 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Dias- tase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	a.m.	mg.	mg.			
July 20-Aug. 7..		{ 125 180	3 13	Negative.	Negative.	Normal amounts with maximal and minimal of six determinations. Morphine sulfate, 2 cc. of 4 per cent solution (80 mg.) subcutane- ously.
Aug. 11.....	8.30					
	10.30	320	16			
" 12.....	10.30	135	12	Negative.	Negative.	
" 13.....	10.30	125	9	"	"	
" 19.....	8.30					Morphine sulfate, 2 cc. of 4 per cent solution (80 mg.) subcutane- ously.
	10.30	419	35	Negative.	1.5percent.	
" 20.....	10.30	132	12			
" 21.....	10.30	139	6			
" 30.....	8.30					Morphine sulfate, 2 cc. of 4 per cent solution (80 mg.) subcutane- ously.
	10.30	402	41			
" 31.....	10.30	114	10	Negative.	1.8percent.	
Sept. 1.....	10.30	138	9			

of blood sugar or of diastatic activity of the blood. Table VI is the protocol of one animal.

Two rabbits were bled repeatedly. The hemoglobin was markedly reduced. Unfortunately the cell counts were not determined at the beginning of the experiment but the figures given indicate a marked

reduction. Associated with the anemia was an increase in blood sugar, as has been found by Hirsch. There was also an increase in diastase as indicated in Table VII.

TABLE IV.

*Effect of the Subcutaneous Injection of Adrenalin Chloride. Rabbit 4.*  
Weight 1.8 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Dias-tase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	a.m.	mg.	mg.			
July 26-Aug. 7..		{ 123 160	3 6	Negative.	Negative.	Normal amounts with maximal and minimal of six determinations.
Aug. 11.....	8.30					
	10.30	446	5			Adrenalin chloride, 2 cc. of 1:1,000 solution subcutaneously.
" 12.....	10.30	124	7	Negative.	2.0 per cent.	
" 13.....	10.30	232	7	"	Negative.	Adrenalin chloride, 2 cc. of 1:1,000 solution subcutaneously.
" 19.....	8.30					
	10.30	579	11			Adrenalin chloride, 2 cc. of 1:1,000 solution subcutaneously.
" 20.....	10.30	162	6	Negative.	1.8 per cent.	
" 21.....	10.30	192	6			Adrenalin chloride, 2 cc. of 1:1,000 solution subcutaneously.
" 30.....	8.30					
	10.30	427	9			Adrenalin chloride, 2 cc. of 1:1,000 solution subcutaneously.
" 31.....	10.30	138	2	Negative.	2.5 per cent.	
Sept. 1.....	10.30	129	4	"	1.7 " "	
" 2.....	10.30	121	4	"	Negative.	

Nine rabbits were definitely asphyxiated either by smothering or by placing them in the Benedict animal calorimeter under low oxygen tension. In one no hyperglycemia was produced; in six moderate hyperglycemia resulted with a tendency to increase in the amount of blood diastase in two. In two marked hyperglycemia with

TABLE V.

*Effect of Puncture of the Floor of the Fourth Ventricle. Rabbit 5.*  
Weight 1 kilo.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Dias-tase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	a.m.	mg.	mg.			
Sept. 14-24.....		{ 120 129	7 9	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
Oct. 4.....	11.45					Bernard puncture of floor of fourth ventricle.
	p.m.					
	1.45	536	8			
" 5.....	1.45	90	10	Negative.	0.5 per cent.	
" 6.....	1.45	110	6	"	Negative.	
" 10.....						Died.

TABLE VI.

*Effect of Diet. Rabbit 6.*

Weight 1 kilo.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1920	mg.	mg.			
July 21.....	155	8	Negative.	Negative.	After green vegetable diet for 5 days.
" 23.....	140	5			After green vegetable diet for 7 days.
" 26.....	168	4			After carrots, oats, and water for 3 days.
" 30.....	137	5			After carrots, oats, and water for 7 days.

a definite increase in the concentration of blood diastase was produced. Table VIII is the protocol of one rabbit.

Total excision of the pancreas was successfully performed in three dogs, one of which lived 36 hours, another about 48 hours, and a third about 56 hours. All showed hyperglycemia, glycosuria, and marked

TABLE VII.

*Effect of Repeated Bleeding. Rabbit 7.*

Weight 0.75 kilo.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1920	mg.	mg.			
Oct. 15-17....	{ 91 114	2 4	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
" 26.....	140	4			After 9 days intermission.
" 27.....	125	7			
" 28.....	100	3			
Nov. 4.....	97	4			After 7 days intermission.
" 5.....	119	3			
" 6.....	115	3			
" 12.....	123	3			After 6 days intermission.
" 13.....	117	7			
" 14.....	121	4			Red blood cells 6,000,000; hemoglobin (Sahli) 55 per cent.
" 18.....	148	15			After 4 days intermission.
" 19.....	144	16			
" 20.....	158	15			
" 24.....	205	10			After 4 days intermission.
" 25.....	205	15			Red blood cells 5,360,000; hemoglobin (Sahli) 55 per cent.
" 26.....	163	13			

TABLE VIII.

*Effect of Asphyxia. Rabbit 8.*

Weight 1.2 kilos.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1921	mg.	mg.			
Jan. 20.....	122	4	Negative.	Negative.	Normal.
" 21.....	107	7			"
" 22.....	81	4			"
Feb. 7.....	347	19			Asphyxiated in Benedict tank; low oxygen ten- sion for $\frac{1}{2}$ hr.
" 8.....			Negative.	1.4 per cent.	

increase in diastatic activity of the blood. The dog illustrated in the protocol (Table IX) was free from general infection, one other showed a slight general fibrinous peritonitis, and one showed gangrene of the duodenum. The results, however, were parallel in all three animals.

TABLE IX.

*Effect of Pancreatectomy. Dog 1.*

Weight 11 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Dias-tase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	a.m.	mg.	mg.			
Nov. 18-22.....		{ 168 179	13 17	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
" 23.....						Complete pancreatectomy; finished 11 a.m.
	11.00	319	17			Blood at close of operation.
	p.m.					
	1.00	241	16			Blood 2 hrs. after operation.
	9.00	365	23	Negative.	2.3 per cent.	Blood 10 hrs. after operation.
	a.m.					
" 24.....	11.00	500	26	"	4.0 " "	Blood 24 hrs. after operation.
" 25.....	11.00	810*	45	"	5.0 " "	Blood 48 hrs. after operation.
" 26.....						Animal found dead in cage.

\* This high figure was determined by identically the same method as the others. The other two animals showed maximum figures for blood sugar of 416 and 540 mg., and maxima for diastatic activity of 53 and 31 mg.

Two dogs were completely anesthetized with ether for 1 hour. A slight increase in blood sugar and in diastatic activity of the blood was found; both of these were of short duration. In one animal



with control figures for diastatic activity of 3 to 13 mg. per 100 cc., the ether anesthesia was followed 10 hours later by a figure of 22 mg. That this is not constant will be seen by reference to the protocol of the animal in which enteroenterostomy was performed (Table XI). The other experiment with ether is shown in Table X.

TABLE X.

*Effect of Ether Anesthesia. Dog 2.*

Weight 10 kilos.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1920	mg.	mg.			
Nov. 7-9.....	$\left\{ \begin{array}{l} 105 \\ 134 \end{array} \right.$	$\left\{ \begin{array}{l} 4 \\ 13 \end{array} \right.$	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations. Before anesthesia.
" 16.....	163	14			
	240	12			
	240	18	No urine.		Blood 2 hrs. following anesthesia.
" 17.....	153	8	Negative.	Negative.	Blood 10 hrs. following anesthesia.
" 18.....	143	5			Blood 24 hrs. following anesthesia.
	137	8			Blood 36 hrs. following anesthesia.
					Blood 48 hrs. following anesthesia.

A dog was subjected to lateral enteroenterostomy. The operation required 1 hour and was followed by an uneventful recovery. Table XI shows that there was a tendency to hyperglycemia but no increase in diastatic activity of the blood.

TABLE XI.

*Effect of Enteroenterostomy Operation. Dog 3.*

Weight 12 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Dias- tase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	a.m.	mg.	mg.			
Dec. 14-16.....		{ 89 109	8 12	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
" 20.....						Enteroenterostomy (1 hr. duration). Blood immediately following operation.
	12.00	222	7			
	p.m.					
	2.00	161	5			Blood 2 hrs. following operation.
	12.00	115	13	Negative.	Negative.	Blood 12 hrs. following operation.
" 21.....	a.m.					
	12.00	119	5			Blood 24 hrs. following operation.
	p.m.					
	12.00	136	4	Negative.	Negative.	Blood 36 hrs. following operation.
" 22.....	a.m.					
	12.00	130	6			Blood 48 hrs. following operation.
" 23.....	12.00	114	12			Blood 72 hrs. following operation.

## IV.

## SUMMARY.

Upon the subcutaneous injection of phlorhizin and uranyl nitrate, their action being checked by the production of glycosuria, no constant change was produced in the concentration of the blood diastase. The adrenalin and puncture of the floor of the fourth ventricle both produced a marked transitory hyperglycemia and glycosuria without altering the content of blood diastase. On the other hand, morphine, when producing a marked definite hyper-

glycemia, caused also a decided increase in the concentration of the blood diastase.

By varying the diet of normal rabbits no material change was produced in the amount of blood diastase. By bleeding normal rabbits periodically as carried out in the experiments, there was no effect upon the diastatic activity until definite hemoglobinemia was produced; then there was a tendency to increase in the diastatic content simultaneously with the rise in blood sugar.

In order to determine the factors leading to the increase in blood diastase by the injection of morphine, the effect of asphyxia was noted. In the asphyxiated as well as in the morphinized animals when marked hyperglycemia was produced there resulted a decided increase in the concentration of the blood diastase. We have no ground for an opinion as to whether or not morphine hyperglycemia is due to asphyxia. All our morphinized animals showed respiratory depression and it appears at least possible that the increase in diastatic activity may have something in common with that of asphyxia.

Upon complete removal of the pancreas in dogs the hyperglycemia and glycosuria were progressive until death and were accompanied by a definite decided increase in the diastatic activity of the blood, the rise occurring at varying intervals following the operation in the different animals; but in no way was the increase in the diastase proportional to the amount of blood sugar.

Except for a slight tendency to increase 12 hours following the anesthesia, the ether anesthesia administered for 1 hour produced no definite change in the diastatic content of the blood, even though a slight hyperglycemia was immediately produced. The control operation performed consisted in an enteroenterostomy of 1 hour's duration, as a result of which the diastatic content was not changed from the normal. The slight hyperglycemia produced was undoubtedly the result of ether anesthesia.

Watanabe points out the great variation in the diastatic activity of the blood of rabbits, which is also found in our experiments. The same applies to the dog. In both animals the normal diastatic activity is greater than that of man.

If it prove true upon more extended study that human diabetes is associated with an increase in diastatic activity of the blood, as

is indicated by the studies of Myers and Killian and of DeNiord and Schreiner, it would seem that the experimental forms of this disease produced by pancreatectomy and perhaps also by asphyxia and morphine administration more nearly approximate the human disease than do other forms of experimental hyperglycemia. It is suggested that when inferences are drawn from experimental studies of animals these similarities and dissimilarities should be taken into consideration.

## V.

## CONCLUSIONS.

1. The diastatic activity of the blood is not materially changed by the subcutaneous injection of phlorhizin, uranyl nitrate, or adrenalin.
2. The diastatic activity of the blood is not affected by puncture of the floor of the fourth ventricle.
3. The diastatic activity of the blood is definitely increased by subcutaneous injections of morphia when a marked hyperglycemia is produced. The same phenomenon appears in asphyxia.
4. Complete pancreatectomy in dogs produces an increase in the diastatic activity of the blood.
5. The diastatic activity of the blood is not altered by the diet.
6. Ether anesthesia, alone, produces a very slight tendency to increase in the diastatic activity of the blood.
7. Repeated periodic small hemorrhages (4.5 cc.) produce no change in the diastatic activity of the blood until a definite color anemia results, when a slight tendency toward increase occurs simultaneously with the rise in blood sugar.
8. The diastatic activity of the blood is not proportional to the concentration of the sugar in the blood.

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## TYPHUS FEVER AMONG RECENT IMMIGRANTS.

### EXPERIMENTAL DEMONSTRATION OF THE IDENTITY OF THIS DISEASE WITH EUROPEAN EPIDEMIC TYPHUS FEVER.

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PLATE 25.

(Received for publication, May 20, 1921.)

In January, 1921, through the kindness of Dr. S. B. Wolbach of Harvard Medical School, we received guinea pigs in which two strains of typhus virus were being propagated. One of these strains was originally derived from the blood taken from a patient during the height of typhus fever; the other, from the saline solution washings of a cage harboring typhus-infected lice. In July, 1920, with these materials, Dr. Wolbach, working in Warsaw, Poland, induced experimental typhus fever in guinea pigs; in the next half year he transmitted it through about fifteen successive animals and determined the existence of a cross-immunity between the human and louse strains.

In February, 1921, when we were engaged in an experimental study of the virus of typhus fever, employing for this purpose the guinea pigs given to us by Dr. Wolbach, a number of immigrants arrived at the Port of New York from different parts of Europe and showed a syndrome of headache, continued fever, petechial rash, positive Weil-Felix reaction<sup>1,2</sup> and infestation with body lice, which was suggestive of typhus fever. Through the kindness of Dr. John E. Holt-Harris, Director of Laboratories at the Quarantine Station, we were enabled

<sup>1</sup> Weil, E., and Felix, A., *Wein. klin. Woch.*, 1916, xxix, 974; 1917, xxx, 1511. This test is based on the observation of Wilson (Wilson, W. J., *J. Hyg.*, 1909, ix, 332; 1910, x, 155) that agglutinins for intestinal, colon-like bacilli are present in the serum of typhus fever patients.

<sup>2</sup> Dr. John E. Holt-Harris, Director of Laboratories at the Quarantine Station, applied the Weil-Felix test to these patients and found definite agglutination reactions in all—indeed, in some instances in dilutions of 1:1,280.

to obtain blood from an immigrant suspected of having typhus fever. We inoculated the blood into guinea pigs in which was set up an experimental disease which we were thus able to compare with the experimental disease being propagated in these animals with the Polish virus.

Herewith is presented a detailed study of the experimental disease induced in the guinea pig by injecting the blood from this immigrant and the results of a comparison of this experimental disease with that set up in the same animal with the human and louse strains of the Polish virus.

#### *Source of Material.*

The patient from whom material was collected for the following experiments was a young, adult female, a native of Czecho-Slovakia, who had embarked for New York at Trieste, Italy. At the time of examination she had been ill for 8 days with headache and continued fever. Her face showed the suffusion and her conjunctivæ the injection characteristic of typhus fever, and the anterior surfaces of her forearms and arms were covered with a fading petechial rash. The Weil-Felix reaction, performed by Dr. Holt-Harris, was positive in a serum dilution of 1:320. Body lice infested the patient before admission to the hospital.

#### *Transmission to Guinea Pigs.*

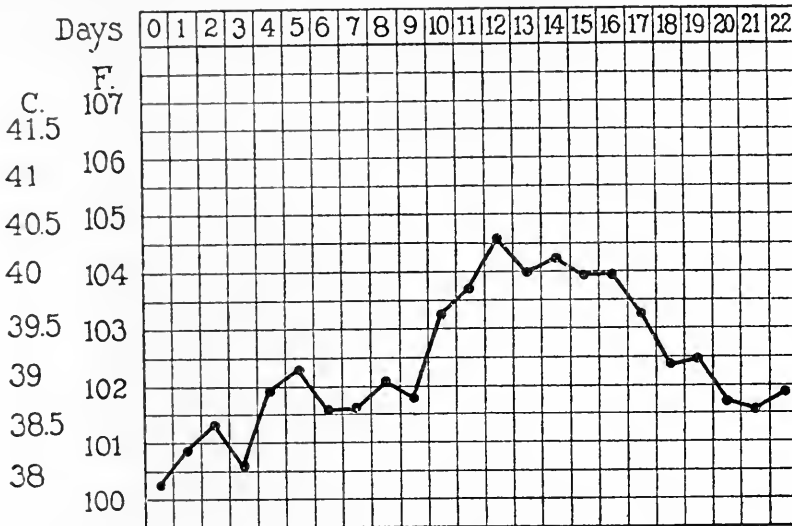
Blood was obtained on the 8th day of illness, by venous puncture with a sterile syringe previously washed in 50 per cent sodium citrate solution, and 3 cc. of the blood, which proved sterile by aerobic cultivation tests, were immediately injected intraperitoneally into each of two guinea pigs, A and B.

*Protocol 1.*—Guinea Pig A, after 19 days during which the average temperature was 38.5°C., showed on the 20th day a rise of temperature to 40.4°C. It was then bled by heart puncture with a needle and syringe prepared in the usual manner and 3 cc. of the blood, sterile by aerobic tests, were injected into each of three normal guinea pigs. The rise of temperature in the second series occurred, on an average, 11 days after injection, when the blood, in the same amount, was transferred to a third group. In this way we have succeeded in transmitting the fever through at least nine successive animals, with an average incubation period of 9 days.



Guinea Pig B showed a rise in temperature to 40.4°C. on the 7th day after injection, when it was bled and the blood transferred to three normal animals. The guinea pigs of this and subsequent passages (second to fourth) yielded profuse growths of *B. proteus* from the heart's blood and this series was, therefore, discontinued and the results were discarded.

The febrile reaction in the guinea pigs which were allowed to recover lasted from 4 to 8 days. Thereafter the temperature promptly fell to normal and the animals remained well (Text-fig. 1).



TEXT-FIG. 1. Fifth guinea pig passage of the disease induced by the blood of the immigrant. Incubation period 9 days; fever 8 days.

The guinea pigs killed during the febrile reaction showed the following pathological picture.

*Macroscopic Examination.*—All the organs were normal in appearance with the exception of the spleen which was slightly enlarged, darker than normally, and of which the lymphoid follicles were prominent and raised above the surface. Occasionally the spleen was not enlarged. The skin, because of its hairiness, showed no definite lesions on its outer surface. The inner surface, however, especially after the corium had been stripped off, revealed occasional petechial spots.

*Microscopic Examination.*—The central nervous system showed characteristic lesions. There was no meningeal involvement except in the case of its blood vessels, which showed the changes described below. The gray matter of the brain, especially that of the midbrain, contained many small, localized hemorrhages. The striking changes were microscopic and consisted of focal accumulations of cells about the capillaries and arterioles. These lesions have been described in

detail by Ceelen,<sup>3</sup> Nicol,<sup>4</sup> and others in European typhus fever, by Doerr<sup>5</sup> and others in the experimental disease in guinea pigs, and recently by Wolbach and Todd<sup>6</sup> in Mexican typhus fever. The nodules consisted mainly of macrophages, or endothelial leucocytes of Mallory,<sup>7</sup> and a few leucocytes, mononuclears, and polymorphonuclears, and were always to be found in close proximity to the vessels. The vessels were surrounded by a more or less dense collar of the macrophages or endothelial leucocytes and leucocytes of the mononuclear and polynuclear type, which at some points was sufficient to form a distinct nodular mass giving rise to the *periarteriolitis nodosa* described by Nicol<sup>4</sup> (Fig. 1). The endothelium of the vessels, often markedly swollen, was necrotic in some portions and proliferated in others; at times the endothelial cells filled the lumen. In other arterioles and capillaries occluding thrombi were sometimes demonstrable.

The heart tissue showed the vascular lesions described by Nicol:<sup>4</sup> localized necrosis of the intima, swelling and proliferation of endothelial cells, thrombus formation, perivascular accumulations of endothelial leucocytes, polymorphonuclears, and mononuclears to form, in some areas, a *periarteriolitis nodosa*, focal nodules of these cells appearing in the interfibrillary spaces usually in proximity to the vessels. Besides, there were a number of small localized hemorrhages (Fig. 2).

The structure of the spleen obscured the vascular picture, but the organ showed, as a rule, congestion and leucocytic infiltration of the splenic pulp, as noted by Nicol.<sup>4</sup> The lymphoid follicles were enlarged and at the center a number of endothelial leucocytes were noted in active phagocytosis, a picture not unlike that seen by Mallory<sup>8</sup> in Peyer's patches in typhoid fever.

The skin showed in the corium nodular areas similar to those found in the brain and heart. These were near the capillaries and arterioles which showed the changes in the endothelium and the thrombus formation described above. In addition there were small, localized hemorrhages (Fig. 3). Similar vascular lesions in the skin of man have been described by Fraenkel,<sup>9</sup> Aschoff,<sup>10</sup> von Chiari,<sup>11</sup> and others in cases of typhus fever in Europe, by Wolbach and Todd<sup>6</sup> in those in Mexico, and by Löwy<sup>12</sup> in experimental typhus fever of guinea pigs.

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<sup>3</sup> Ceelen, *Berl. klin. Woch.*, 1916, liii, 530.

<sup>4</sup> Nicol, K., *Beitr. path. Anat. u. allg. Path.*, 1919, lxv, 120.

<sup>5</sup> Doerr, R., *Centr. Bakt., 1te Abt., Orig.*, 1921, lxxxv, Nos. 6 and 7, p. 2.

<sup>6</sup> Wolbach, S. B., and Todd, J. L., *Ann. Inst. Pasteur*, 1920, xxxiv, 153.

<sup>7</sup> Mallory, F. B., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 69.

<sup>8</sup> Mallory, F. B., *J. Exp. Med.*, 1898, iii, 611; *The principles of pathologic histology*, Philadelphia and London, 1920, 170.

<sup>9</sup> Fraenkel, E., *Münch. med. Woch.*, 1914, lxi, 57; 1915, lxii, 805.

<sup>10</sup> Aschoff, L., *Med. Klin.*, 1915, xi, 798.

<sup>11</sup> von Chiari, R. F., *Wien. klin. Woch.*, 1917, xxx, 1479.

<sup>12</sup> Löwy, O., *Wien. klin. Woch.*, 1916, xxix, 547.

The kidney, adrenals, liver, and testicles revealed no remarkable changes other than the characteristic microscopic nodular formation and the vascular changes. The lesions in these organs correspond to those found in man, in the kidney by Aschoff,<sup>10</sup> in the adrenals by Nicol,<sup>4</sup> in the liver by Ceelen,<sup>3, 13</sup> and in the testicles by Schmorl.<sup>14</sup>

To summarize the results of the experiments described above, it is shown that the blood of the patient at the Quarantine Station induced in guinea pigs a characteristic febrile reaction of from 4 to 8 days duration, preceded by an average incubation period of 9 days. The disease of which this is the expression could be transmitted through at least nine successive animals. Furthermore, the guinea pigs killed during the height of the reaction revealed on microscopic study of the organs constant pathological changes similar to those found by several investigators of typhus fever of man in Europe and Mexico, and of the experimental disease of the same animal.

In these respects, the experimental disease which we have induced in guinea pigs corresponds to that set up in the same species with the human and louse strains of the Polish virus.

#### *Immunity Experiments.*

Additional evidence in establishing the identity of the experimental disease in guinea pigs induced with the blood of the immigrant with the disease propagated in these animals with the Polish virus, is afforded by means of the immunity reactions. The study on immunity was made from these view-points: (a) the specific immunizing effect of the virus derived originally from the blood of the immigrant when followed by subsequent injections of the active agent of the same disease, and (b) cross-immunity between this disease and that of guinea pigs originally infected with the Polish typhus fever virus of man and lice.

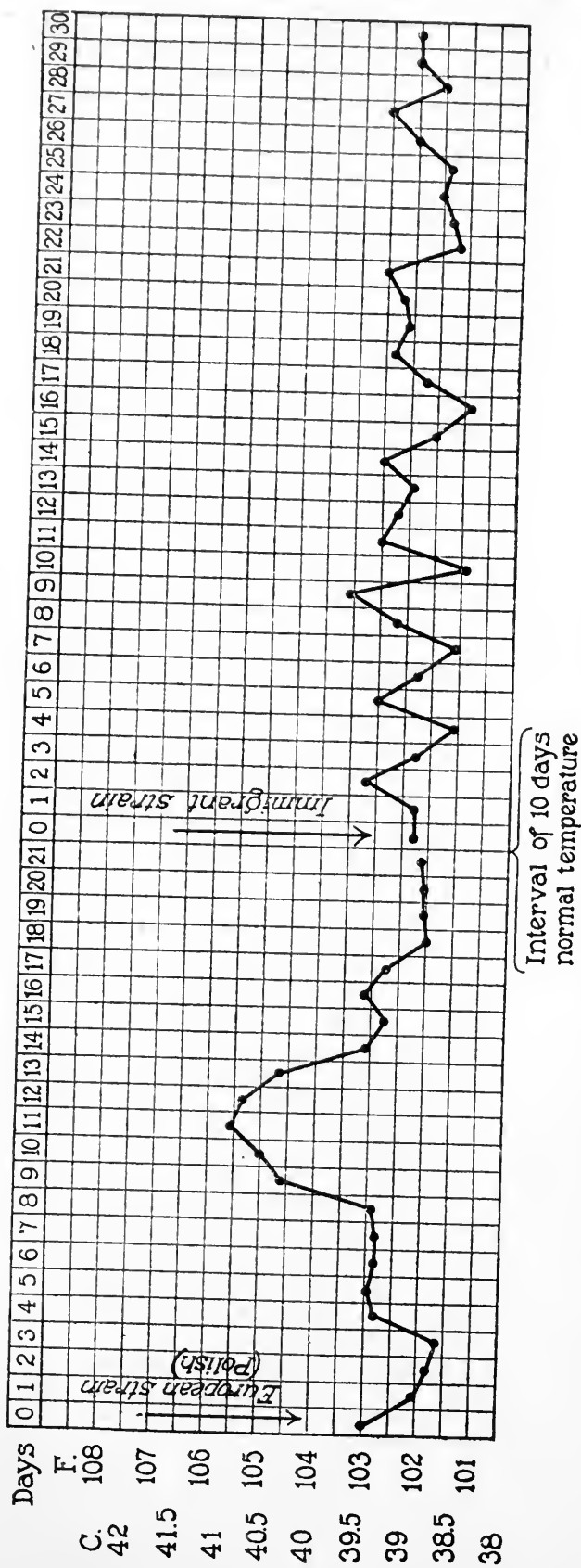
The following protocols are illustrative.

*Protocol 2.*—Guinea Pig B was injected intraperitoneally with 3 cc. of blood from Guinea Pig A, the fourth passage of the experimental disease in this species, derived originally from the blood of the immigrant. After an incubation period

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<sup>13</sup> Ceelen, W., *Med. Klin.*, 1916, xii, 402.

<sup>14</sup> Schmorl, C. G., quoted by Nicol.<sup>4</sup>



TEXT-FIG. 2. This guinea pig, after reacting typically to an intraperitoneal injection of the Polish typhus fever virus, human strain, remained immune to a second injection of the immigrant disease.

of 9 days, Guinea Pig B showed a febrile reaction lasting for 6 days; thereafter the temperature was normal. A control guinea pig, injected similarly, reacted with a rise of temperature after 9 days incubation and was killed on the 1st day of the fever. It showed the typical pathological picture already described. Guinea Pig B after 6 days of normal temperature was reinjected intraperitoneally with 3 cc. of the blood from an animal in the sixth passage of the experimental disease. There was no reaction during a period of observation lasting 1 month. A control animal was inoculated at the time of the second injection of Guinea Pig B with similar material. This animal reacted characteristically after an incubation period of 10 days.

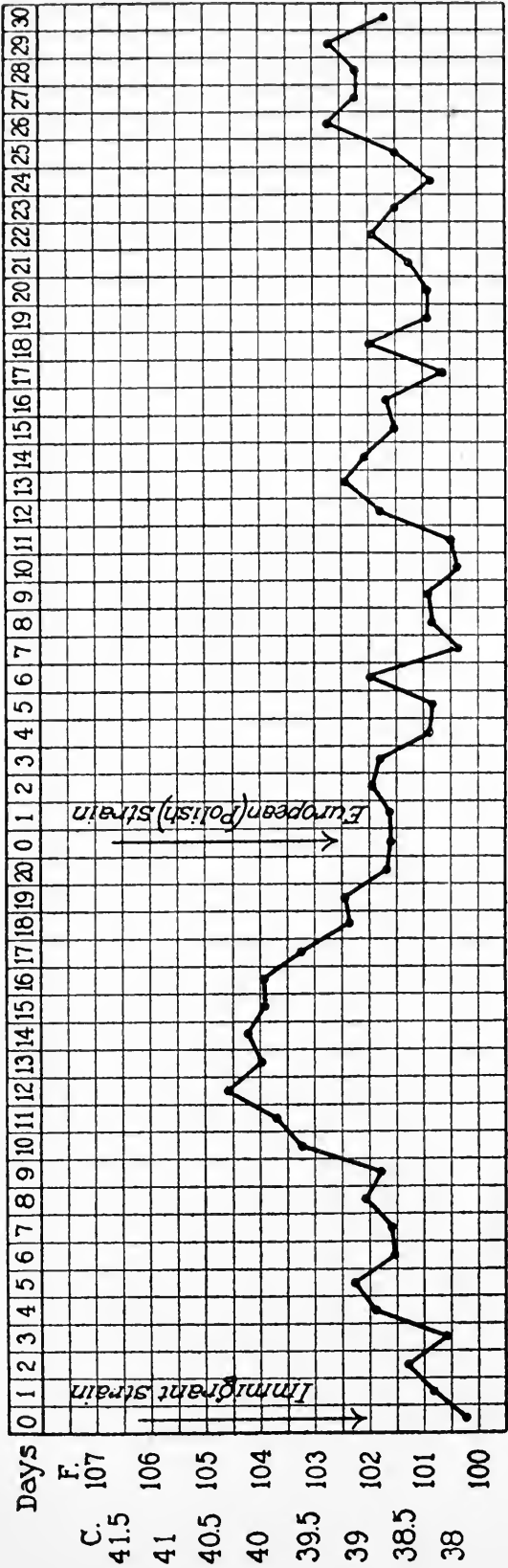
This experiment indicates that after an attack of the disease induced in guinea pigs with the blood of the immigrant, the animal is refractory to subsequent injections of active blood derived through passage from the same patient.

*Protocol 3.*—The following experiments were controlled in that whenever active blood was employed, it was injected into control animals under the same conditions as the test animal, to determine its clinical and pathological action.

Guinea Pig A (Text-fig. 2) was injected intraperitoneally with 3 cc. of blood from a guinea pig in the nineteenth passage of the experimental disease induced with the Polish strain of typhus virus. After an incubation period of 7 days a rise in temperature occurred, lasting for 5 days. Thereafter the animal showed normal temperature for 19 days, after which it was reinoculated with 3 cc. of blood from a guinea pig in the third passage of the experimental disease induced originally with the blood of the immigrant. There was no effect during a period of observation lasting 1 month.

Guinea Pig B was inoculated similarly, but with the eighteenth passage of the Polish typhus virus, louse strain. A 6 day fever following an 11 day incubation period resulted. 2 days after the fever subsided, the animal was reinjected with 3 cc. of blood from a guinea pig in the second passage of the experimental disease induced with the blood of the immigrant. No reaction occurred although the animal was kept under observation over a month.

Guinea Pigs C (Text-fig. 3) and D had reacted characteristically to injections of blood from guinea pigs in the fourth and fifth passages, respectively, of the experimental disease induced with the blood of the immigrant. Guinea Pig C, 4 days after the fever subsided, was reinjected with the blood from a guinea pig in the twenty-sixth passage of the Polish typhus virus, human strain; Guinea Pig D, 3 days after the fever subsided, with the blood of the twenty-fifth passage of the Polish typhus virus, louse strain. Neither animal was affected during a period of observation of over 1 month.



TEXT-FIG. 3. This guinea pig, after reacting typically to an intraperitoneal injection of the blood from a guinea pig in the fourth passage of the disease originally derived from the immigrant's blood, remained immune to a second injection of Polish typhus fever virus, human strain.

Protocol 3 shows that guinea pigs reacting characteristically to the known strains of typhus virus originally derived from a typhus fever patient in Poland, and from typhus-infected lice collected in that country, fail to respond to a subsequent injection of active blood from guinea pigs with the experimental disease set up with the blood from the Czecho-Slovakian immigrant at the New York Quarantine Station. Conversely, guinea pigs reacting typically to the injections of blood from guinea pigs in the line of transmission of the experimental disease induced with the immigrant's blood, are refractory to later injections of blood containing either human or louse strains of the Polish typhus fever virus.

#### DISCUSSION.

The experimental disease induced in guinea pigs with the human and louse strains of the Polish typhus virus is characterized by the following conditions: (a) Transmissibility of the virus from patient to guinea pig and from guinea pig to guinea pig indefinitely. (b) Specific pathology of the affected animals during the height of the reaction. The macroscopic examination reveals no changes in the organs with the exception of enlargement of the spleen<sup>15</sup> and a petechial rash in the deeper layers of the skin. The lesions in the different organs are demonstrable by histological study and consist of a particular vascular change, especially about the blood vessels of the brain. (c) Absence of concomitant or secondary infections by ordinary bacteria (pneumonia, peritonitis, abscesses, pseudotuberculosis, or other diseases, or the presence of these bacteria in the blood). (d) Specific immunity reactions. A guinea pig after reacting to an

<sup>15</sup> It has been stated that the organs in man (Nicol<sup>4</sup>) and in the guinea pig (Doerr<sup>5</sup>) show no macroscopic lesions whatever. But that the spleen is definitely enlarged has been our experience and this is confirmed by the following reports, both in regard to typhus fever in man and experimental typhus fever in animals: Ricketts, H. T., and Wilder, R. M., *J. Am. Med. Assn.*, 1910, liv, 463; Curschmann, H., Typhoid and typhus fever, Philadelphia, 1902, 526; Plotz, H., Olitsky, P. K., and Baehr, G., *J. Infect. Dis.*, 1915, xvii, 1; Nicolle, C., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1916, ix, 127; Grzywo-Dabrowski, W., *Virchows Arch. path. Anat.*, 1918, ccxxv, 299; Danielopolu, D., Le typhus exanthematique, Bucharest, 1919, 222; Shattuck, G. C., Typhus fever with particular reference to the Serbian epidemic, Cambridge, 1920, 111, and others.

injection of the virus remains immune to a subsequent inoculation of the typhus fever virus.

It appears then that the blood from the immigrant could induce in guinea pigs a similar experimental disease, for, as will be noted in the above experiments, it is characterized by (a) transmissibility, (b) particular pathological effects, (c) absence of concomitant or secondary infections by ordinary bacteria, and (d) specific immunity reactions. From the experiments on immunity we infer that the strain of virus inducing the typhus fever occurring last year in Warsaw, Poland, in epidemic form, and that inducing the disease in immigrants this year at the Port of New York, are identical.

#### CONCLUSION.

It is experimentally demonstrated in the guinea pig that the blood from an infected Czecho-Slovakian arriving at the Port of New York from Italy contained the virus of typhus fever and that this strain is identical with the epidemic virus present in man and louse in Poland.

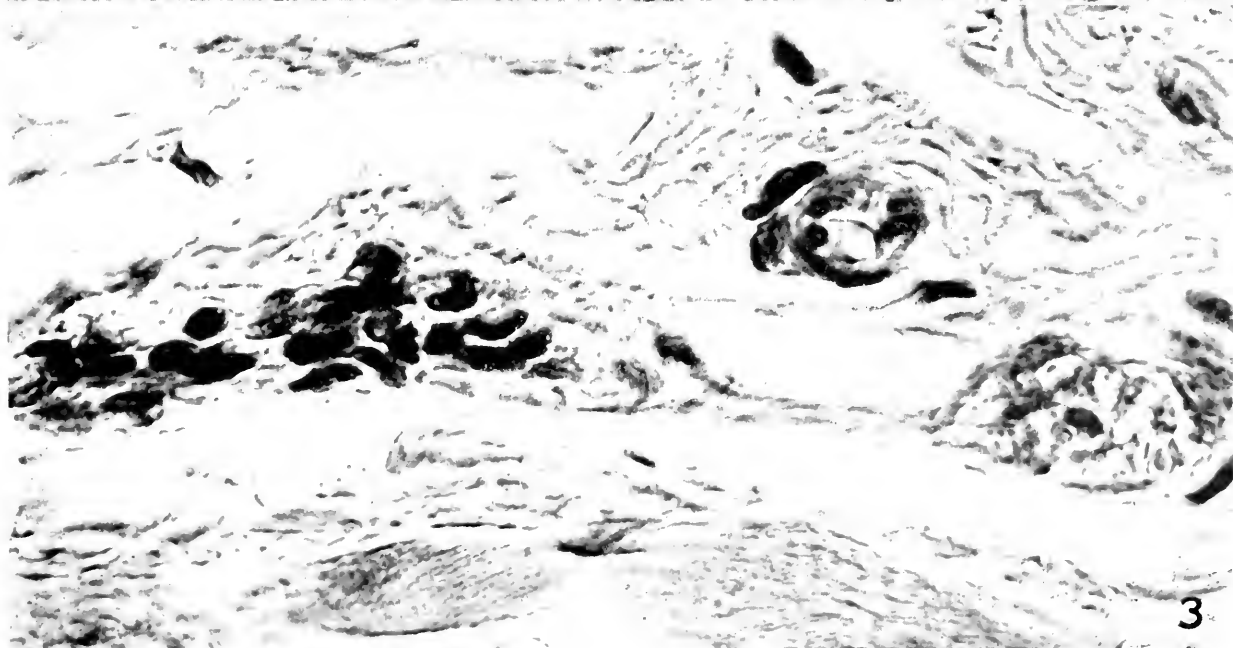
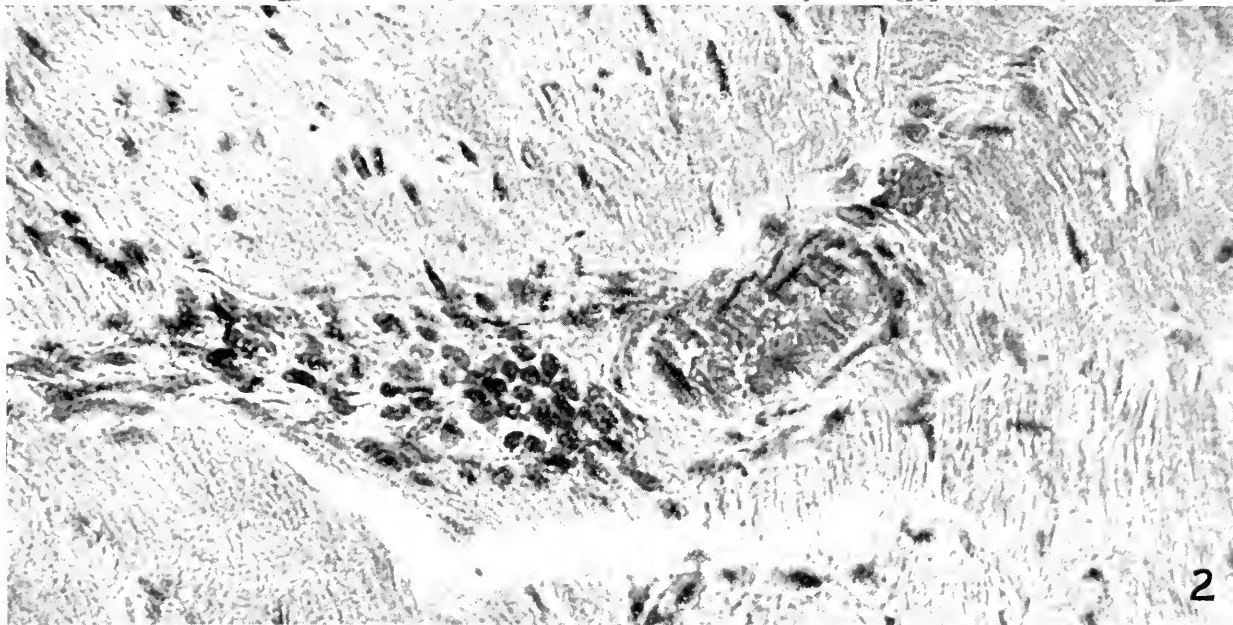
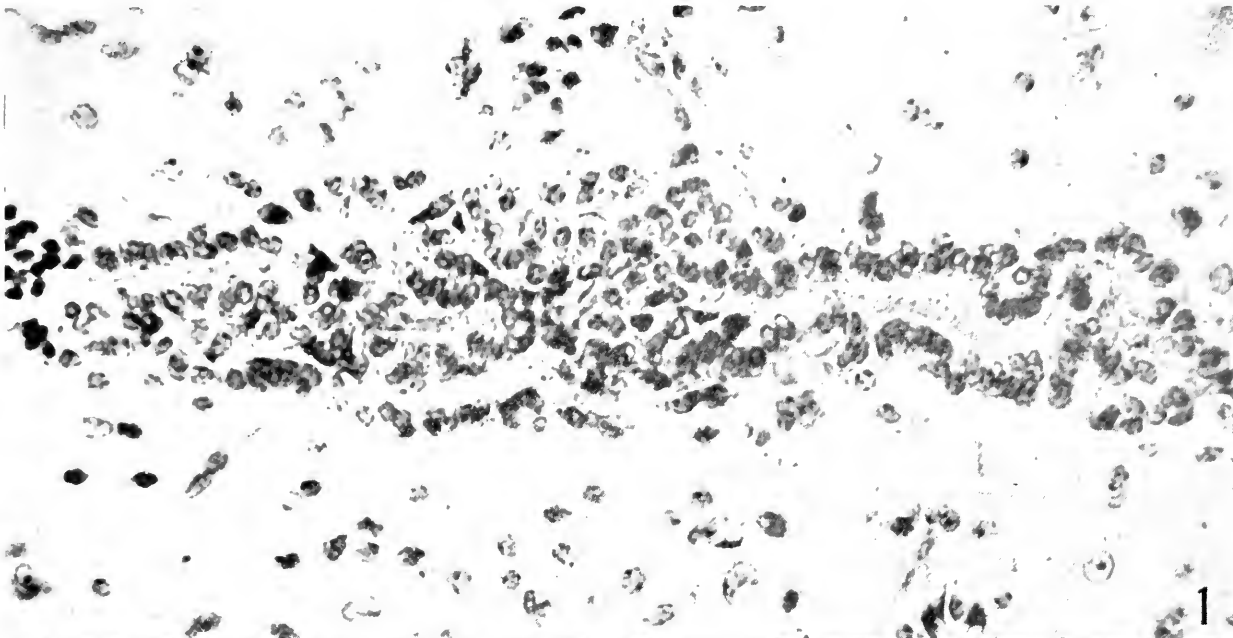
#### EXPLANATION OF PLATE 25.

FIG. 1. Section of brain of guinea pig experimentally infected with the blood from the immigrant. The vascular lesions are shown in detail. There are circumscribed proliferation and accumulation of macrophages and leucocytes about an arteriole, and at one point, a *periarteriolitis nodosa*.  $\times 400$ .

FIG. 2. Section of heart muscle from the same guinea pig. The vascular lesions are shown in detail. They resemble those found in the brain and demonstrated in Fig. 1.  $\times 400$ .

FIG. 3. Section of the corium of the skin from a guinea pig experimentally infected with the blood from the immigrant (fifth guinea pig passage). The circumscribed accumulation of macrophages and leucocytes close to vessels, which show proliferation and swelling of the endothelial cells, is noteworthy. A thrombus is seen in one of the vessels.  $\times 1,000$ .





(Olitsky: Typhus fever among recent immigrants.)



## EXPERIMENTAL STUDIES ON INFLAMMATION.

### I. THE INFLUENCE OF CHEMICALS UPON THE CHEMOTAXIS OF LEUCOCYTES IN VITRO.

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Inflammatory processes usually, if not always, are the result of reaction to chemical substances. Some substances cause severe reactions when in very low concentrations, while others, even in high concentrations, cause little or no inflammation. As far as we can learn, no systematic study has ever been made to determine what chemical, physical, or physiochemical properties a substance must have in order that it may incite inflammation, or that it may be unable to incite inflammation. Can definite laws be discerned governing the capacity of chemical substances to stimulate inflammatory reactions? Is it possible to predict from the chemical or physical properties of a substance its capacity for inflammatory stimulation? Such information, if it can be obtained, should be of great value in chemotherapeutic work, since it might be applied to reducing the inflammatory reaction produced by drugs that must be injected into the tissues, and hence this study was undertaken at the suggestion of Dr. H. Gideon Wells as part of the program of chemotherapeutic investigation that is being conducted in the Otho S. A. Sprague Memorial Institute.

In studying this problem it is necessary to consider the several components of inflammatory reaction; namely, vascular changes, migration of cells and fluids, cell injury, and cell proliferation. In this paper will be considered only one phase of the work, the influence of chemicals upon the chemotaxis of leucocytes *in vitro*, a subject which has already received not a little investigation.

Most of the literature on this subject has been fully reviewed by Wells,<sup>1</sup> who has discussed the subject from the earliest studies of Engelmann who worked with protozoa in 1881, up to the time of Jacques Loeb, who worked with chemical tropisms. This may be briefly summarized in the following lists of the substances supposedly positive, negative, and neutral as chemotactic agents for leucocytes.

Various authors have stated that the strongly positively chemotactic substances are: sterilized and living cultures of bacteria, whether pathogenic or non-pathogenic; papayotin or papain (in rabbits) (Gabritchevsky<sup>2</sup>); carbolglycerol extracts of different viscera and tissues; egg albumin, gelatin, peptone, and alkali albuminate; metallic copper, iron, mercury, and their salts;<sup>3</sup> and substances soluble in lipoids in extreme dilution (Hamburger<sup>4</sup>).

Among the supposedly negatively chemotactic substances the following have been mentioned by various authors: concentrated solutions of sodium and potassium salts; lactic acid in all concentrations; quinine (0.5 per cent); alcohol (10 per cent); chloroform, in watery solution; jequirity (2 per cent, passed through a Chamberland filter); glycerol (10 to 1 per cent); bile; *B. cholerae gallinarum* (Gabritchevsky); mercury perchloride, cholesterol, arsenic, antipyrine, acetic acid; concentrated solutions of calcium, sodium, and potassium salts; alcohol in over 10 per cent concentration, chloroform, bile, glycerol in concentrations of 1 to 10 per cent; and atophan, or phenylcinchonic acid.

Among the neutral or indifferent agents have been mentioned water; calcium and sodium salts in concentrations of 0.1 to 1 per cent; phenol; phlorhizin; papayotin (in frogs); glycogen; peptone; blood; hemoglobin; uric acid up to 2 per cent; and bouillon.

These are only tentative statements, for many contradictory results have been obtained, and it is almost impossible to avoid some experimental errors.

The most important of the later work has been done by Hamburger, and his coworkers, on phagocytosis. This bears a close relation to chemotaxis and must be considered at the same time as chemotaxis. Hamburger<sup>4</sup> has shown that "calcium *per se*," as a substance causes a great rise in phagocytosis; it seems to be the only substance acting in this way, apparently aiding chemotaxis. He has investigated this problem from two view-points, *in vivo* and *in vitro* work both being done. In the *in vitro* work, horse leucocytes from citrated serum were transferred to different media and carbon particles added. The

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<sup>1</sup> Wells, H. G., Chemical pathology, Philadelphia, 4th edition, 1920, Chapter XI.

<sup>2</sup> Gabritchevsky, G., *Ann. Inst. Pasteur*, 1890, iv, 346.

<sup>3</sup> Bloch, G., *Centr. allg. Path. u. path. Anat.*, 1896, vii, 785.

<sup>4</sup> Hamburger, H. J., *Physikalisch-chemische Untersuchungen über Phagozyten. Ihre Bedeutung von allgemein biologischem und pathologischem Gesichtspunkt*, Wiesbaden, 1912.

percentage of these particles taken up by the leucocytes was used as a measure of the degree of phagocytosis. The *in vivo* work was done by injecting calcium-free and calcium-containing cultures of bacteria (*B. coli*) under the skin in capillary tubes, and then comparing the size of the resulting leucocyte columns in the calcium-free and calcium-containing cultures. Besides the work on calcium, Hamburger has tested many organic and inorganic compounds. He found that, under the conditions of his experiments, hemoglobin had no bad effects on phagocytic activity no matter what concentrations were employed. Collařgol and other colloidal silver compounds had no effect up to 0.02 per cent, but above that concentration these compounds were injurious and the injury increased in proportion to the concentration. Quinine was injurious to leucocytes, seeming to act upon the cells themselves. Of the halogens, iodine is more poisonous than bromine; fluorine is a strong poison; iodoform increases phagocytosis; mercury, strontium, and barium are indifferent. All fat solvents in small amounts increase phagocytosis; those used were chloroform, ether, chloral, benzene, camphor, some fatty acids, and turpentine. Citrate and sulfite ions act as a poison, preventing any phagocytosis. Oxygen acts like apnea, preventing phagocytosis; carbon dioxide increases phagocytosis. Potassium cyanide has about the same action as carbon dioxide, an increase followed by a decided decrease in phagocytic activities, and then death of the cells.

#### *Method.*

In my work, Wright's method has been employed.<sup>5</sup> Equal volumes of blood must be disposed over equal areas of surface, and come into contact with equal concentrations of the desired reagent throughout. This may be done in the following ways: The first method is to paint hot paraffin containing the desired reagent upon glass slides until 1 mm. thick, then punch circles in the plastic paraffin with a sharp cork-borer, and prize out the discs and clean the glass floor with xylol. The second and preferable method is to use agar in the place of paraffin. Here different concentrations of the drugs desired are mixed with 2 per cent salt-free water solution of agar, then cast into tablets 1 mm. thick, in molds constructed of a pair of glass slides held apart at the ends by pieces of slides 1 mm. thick. The slide which forms the lid of the mold is pushed aside, while the agar is poured in, then brought into position. When this melted agar has set the lateral supports and the lid are removed and circles are cut in the agar with a sharp cork-borer, the discs removed,

<sup>5</sup> Wright, A. E., *Lancet*, 1918, i, 129.

and the cells thus obtained placed upon slabs of 2 per cent salt-free water solution of agar, or upon slabs containing the same concentration of the reagent as contained in the cell. In this way, a window is formed, with the floor and sides of agar containing the desired concentration of the reagent. Two different forms are used. In one, the floor consists of 2 per cent salt-free water solution of agar, the sides of the 2 per cent solution of agar containing the desired reagent. In the other, the floor and sides of the cell contain the same concentrations of the substances to be tested. These different cells are then placed upon a glass slide so that each slide contains from eight to twelve cells, each one of which has a different concentration of the reagent. On each slide there is a control cell, the floor and sides being made of the 2 per cent salt-free water solution of agar.

The slides containing the agar slabs were kept in moist chambers made of Petri dishes whose bottom and sides were lined with moist cotton, so that no water would be lost by evaporation from the agar, thus changing the concentrations. The Petri dishes were kept in warm water, the slides thus being heated to 37°C. and kept at this heat so that the temperature would be the same in each window when the blood was run in and all the time after pouring. Equal amounts of blood were then run into the cells thus made. A capillary pipette was filled with blood as soon as the vein was cut, and 2 drops of this uncoagulated blood were allowed to drop into each window immediately so that there would be no chance of clotting. All the windows were filled at one time so that there would be no differences in the blood itself to be taken into consideration. The dishes containing the blood-filled windows on the slabs of agar were put in the incubator and kept at 37°C. for 45 minutes. After incubation, the clots of blood were washed off with warm sodium chloride solution, 0.9 per cent sodium chloride in distilled water, kept at 37°. If the clots were extremely adherent to the sides, the edges were loosened by touching them gently with a probe, or lifted out with fine forceps. A solution of saturated mercuric chloride could then be poured into the windows to fix the leucocytes if desired. The number of leucocytes adhering to the sides and the floor were then counted under the low power (No. 3) of the microscope.

In this work repeated duplicate tests were made with the blood of the same animal. The number of cells migrating into the agar was always the same within the limits of experimental error, the difference never being more than 25 to 50 cells out of a total of 2,000 to 2,500. The same percentage of the total number of cells always adhered to the control slide; *i.e.*, about 4 per cent. The exact number varied with the degree of leucocytosis but the relative number was always approximately the same.

From 0.0001 to 10 per cent molecular concentrations of the different salts were employed, so that the range of chemotaxis might be studied in relation to the per cent of the salt itself, as well as the positive and negative ions composing the salt, as, for example, NaCl as a salt and Na as an ion as compared with Na in  $\text{Na}_2\text{CO}_3$ , or  $\text{Na}_2\text{S}_4\text{O}$ , and Cl as an ion as compared with Cl in KCl or  $\text{CaCl}_2$ , etc. In some cases a high concentration showed a marked negative chemotaxis, while a low concentration showed a positive one. Whenever possible, neutral or nearly neutral substances were used, so that the reaction would not interfere with the chemotaxis. If, however, the chemical had an acid or alkaline reaction strong enough to give irregular and peculiar results, sodium phosphate as the tri-, di-, or monobasic salt was added in just sufficient concentrations to give a neutral reaction with phenolsulfonephthalein ( $\text{pH}=7.7$ ), and the results were then compared with that of the original chemical alone.

For each substance used, at least four different tests were made with human blood, five with rabbit blood, and two or three, when possible, with guinea pig and dog blood, so that the mean of the several bloods of each species might be used as the final count, providing there was little individual variation. Since the degree of leucopenia or leucocytosis made a great difference in the exact number of leucocytes adhering, though not the relative number, as a routine matter white cell counts were made on the blood of all animals every week, since it was found that in health there is little variation in that length of time. It was found that human blood varied markedly before and after eating, so certain times after eating were chosen for drawing blood when possible, usually  $2\frac{1}{2}$  to 3 hours after a meal.

An interesting observation is that immediately after eating, within 30 minutes, the leucocytes stick much more readily to the



agar than at other times. This holds good for all the species tried. This has been noted for leucocytes on glass slides by several investigators. Cohnheim, among others, mentions the difference in behavior of leucocytes at different times of the day, but the reasons for this have never been shown.

The experimental animals were kept from food for about 3 hours before use. When possible the same animals were used with the different drugs. It was found that with a 2 per cent solution of salt-free agar about 4 per cent of the leucocytes would always adhere to the agar. This was constant with the blood of all the animals employed.

The following chemicals were used:

1. Calcium salts, as the chloride, carbonate, tartrate, citrate, lactate, sulfate, cyanide, and cinnamate.
2. Sodium salts, as the chloride, carbonate, tartrate, citrate, lactate, sulfate, cinnamate, and tri-, di-, and monobasic phosphates.
3. Potassium chloride, acid carbonate, and acid phosphate.
4. Barium sulfate and sulfide.
5. Strontium chloride.
6. Magnesium sulfate, tartrate, citrate, and these compounds mixed with strontium salts.
7. Mercury chloride, nitrate, subsalicylate, and iodate.
8. Organic acids, as oleic, lactic, uric, and nucleinic.
9. Amino-acids and amines, as tyrosine, histidine, glycine, alanine, glutamic acid, aminovalerianic acid, tyramine, and histamine.
10. Alkaloids, as morphine and morphine sulfate, hydrochloride, nitrate, and citrate; codeine and codeine hydrochloride; heroine and heroine hydrochloride; caffeine sulfate, quinine sulfate, strychnine sulfate, and brucine sulfate.
11. Miscellaneous substances, as papayotin, or papain, urea, urethane, creatinine, ethyl alcohol, chloral hydrate, cantharidinum, aspirin (acetyl-salicylic acid), chloretone, glucose, "mustard gas" (dichloroethyl sulfide) "parazol" (dinitro-dichlorobenzene), olive oil, scarlet R (purified), turpentine, veronal (diethylmalonylurea), yeast vitamine (Harris), acetanilide, and ether.

#### EXPERIMENTAL.

##### *Calcium Salts.*

All calcium salts were positively chemotactic for leucocytes except calcium citrate. This was neutral in a 1 per cent molecular concen-



tration, but all other concentrations were negatively chemotactic, the citrate ion overpowering the calcium ion.

Calcium chloride showed a marked increase in the number of cells adhering to the agar with which it was combined, as compared with the control. When there was more than 2 per cent molecular concentration, there was a precipitate through which the light could not penetrate, so that the cells could not be counted when the floor was composed of agar containing the same concentration of the salt as the margins.

An illustrative series of experiments with calcium chloride is given in detail below. The figures in the top row are the percentage of calcium chloride contained in the water agar solution of each cell, and this represents the molar or gram-molecular concentration of calcium in calcium chloride. Thus in the case of a 5 per cent solution 2.75 gm. of calcium chloride would be used with 20 cc. of a 2 per cent solution of water agar, so that there would be 1 gm. of calcium present in 20 cc. of the agar solution. An exact count was made of the number of cells migrating into the agar and adhering, after the clot had been removed and the window and floor of the cell washed with normal salt solution, and this is recorded in each of the remaining rows. Each row gives results obtained with a separate sample of blood. The lowest row of figures in each group is the mean of the other figures in that column.

In the second group of figures, from observations with the floor as well as the margins of the cell formed of agar plus the different concentrations of calcium chloride, it will be seen that many more cells adhere in concentrations of 0.5 to 2 per cent than when only the margins of the cell contained calcium chloride. When frozen sections were made of these different agar plates it was found, after examination of the serial sections, that the leucocytes migrated into the agar to the depth of 0.68 to 0.84 mm. when calcium chloride was combined with the agar, while the migration into the plain agar occurred only to the depth of 0.22 to 0.47 mm. when the margins contained the calcium chloride.

*Calcium Chloride Combined with Agar. Floor of 2 Per Cent Plain Agar. Human Blood.*

	5 per cent.	3 per cent.	2 per cent.	5 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.08 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	Control.
	2,108	3,149	2,508	3,097	3,146	3,564	4,036	4,587	4,562	3,736	2,836	2,564	2,573	1,892
	1,872	2,569	3,095	2,764	3,593	4,095	4,539	3,842	4,594	3,874	2,893	2,482	2,531	1,936
	1,869	2,546	3,148	3,056	3,582	4,082	3,584	3,792	4,327	2,746	2,683	2,538	2,443	2,180
	2,304	2,492	3,345	3,543	4,726	5,238	5,098	5,070	4,956	4,086	3,082	2,775	2,132	2,038
	2,495	3,178	3,487	3,642	4,946	5,654	5,187	5,136	5,032	4,301	3,149	2,867	2,845	2,645
	1,657	3,482	3,542	5,035	5,692	5,237	5,109	5,192	5,017	4,785	3,376	2,850	2,742	2,543
Average	2,051	2,901	3,187	3,523	4,260	4,645	4,591	4,603	4,746	3,921	3,003	2,696	2,546	2,205

*Floor of 2 Per Cent Agar Plus Calcium Chloride. Human Blood.*

	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.08 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	Control.
	8,324	7,891	8,513	7,549	4,079	5,538	3,175	2,486	2,568	3,125	3,146
	5,876	5,683	5,962	5,872	3,762	4,163	2,104	1,973	2,015	2,047	1,985
	5,769	5,468	5,870	5,637	3,652	4,075	2,346	2,074	2,016	2,168	2,042
	5,678	5,469	6,085	5,364	3,869	4,197	2,834	1,985	2,175	2,068	2,164
	5,697	5,683	6,197	5,639	3,749	4,329	2,096	2,438	1,986	2,157	2,352
	6,107	5,789	5,985	6,097	3,578	4,097	2,189	2,357	2,439	2,146	2,458
Average . . . . .	6,242	5,997	6,452	6,115	3,761	4,396	2,457	2,235	2,206	2,260	2,356

Calcium tartrate was slightly positively chemotactic, there being an increase of 25 per cent in the number of cells adhering at 0.25 per cent molecular concentration, as compared with the control.

Calcium lactate was slightly positively chemotactic despite the negative influence of the lactate ion, there being an increase of 30 per cent over the control at 0.1 to 0.05 per cent molecular concentration.

Calcium cyanide was slightly positively chemotactic when the floor of the cell consisted of plain water solution of agar and the sides of the window consisted of 2 per cent water solution of agar plus calcium cyanide. When both the floor and the sides of the

cell were made of the agar containing the cyanide, there was a slightly negative chemotaxis. This may possibly be explained on the basis that calcium cyanide, except in minute quantities, is highly toxic, and only in these small quantities does the chemotactic influence of the calcium overcome the cyanide poisoning. It has been because of these variations in action that the plain slab and the salt-containing slab have been used in every case. This will be referred to later. The average figures obtained in five experiments with calcium cyanide and human blood are given below:

*Calcium Cyanide.*

1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
3,614	3,548	4,018	3,564	3,532	3,147	2,591	1,403	2,017
—	—	2,018	2,136	547	536	241	218	2,106

In all the following tables, the first row of figures will be the average of five experiments for those cells in which the floor of the cell consisted of salt-free water solution of agar, the lower rows of figures will be for those cells in which both the floor and walls were composed of agar containing the tested reagents. Because of space restriction only a very small proportion of the actual results are published, for the purpose of illustrating the procedure.

Calcium carbonate, calcium sulfate, and calcium cinnamate were all somewhat positively chemotactic and varied very little from each other. The greatest number of leucocytes adhering was when the molecular concentration was 0.25 per cent, there being an increase of 50 per cent over the control.

Calcium citrate was negatively chemotactic in all concentrations over or below 1 per cent, as mentioned previously. A typical set of average figures for human blood with calcium citrate follows:

*Calcium Citrate.*

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
1,507	2,464	3,092	2,573	2,798	1,549	1,485	1,502	3,017
1,673	2,793	3,052	2,489	2,538	1,537	1,426	1,507	3,164

Sodium Salts.

The sodium salts had no constant action that depends on the sodium ion *per se*. Sodium chloride was positively chemotactic at 1, 0.8, and 0.75 per cent molecular concentrations, nearly twice as many leucocytes adhering as in the control slides with the blood of the same animal. In more than 5 per cent molecular concentration there was a negative chemotaxis, only two-thirds to one-half as many cells adhering as in the control slides. These figures applied to all blood, human, rabbit, dog, and guinea pig. A typical set of figures obtained in a series of experiments with sodium chloride is shown below:

Sodium Chloride.

7.5 per cent.	5 per cent.	4 per cent.	3 per cent.	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	Control.
1,042	1,965	2,386	2,576	3,091	4,027	3,957	2,538	2,273	2,015	1,869
1,036	1,538	2,069	2,537	3,096	4,586	4,097	2,437	2,097	2,175	2,013

Sodium sulfate was neutral in regard to chemotaxis in all concentrations below 4 per cent molecular concentration for all animals. Above this concentration, it was negatively chemotactic. Sodium carbonate was neutral in almost all concentrations, although with from 0.8 to 1.2 per cent molecular concentration there was a slight positive chemotaxis. Sodium tartrate was neutral for almost all concentrations. Sodium citrate was neutral in all concentrations below 0.1 per cent; above this it was negatively chemotactic for human blood; for rabbit blood there was a slight degree of negative chemotaxis for molecular concentrations above 0.5 per cent.

Sodium Citrate.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
Human blood.							
1,579	1,468	1,452	1,792	2,247	2,296	2,158	2,375
1,538	1,396	1,425	1,658	2,153	2,153	2,096	2,436
Rabbit blood.							
2,548	2,459	2,197	2,987	3,084	3,178	3,097	2,976
2,356	2,351	2,146	2,857	2,967	3,089	3,096	2,963

Sodium lactate was negatively chemotactic in all concentrations for human blood, but with rabbit blood there was no negative chemotaxis, and with the blood of three of the five animals, there was an increase of about 25 per cent in the number of leucocytes adhering at 0.05 to 0.1 per cent molecular concentration.

Sodium cinnamate was somewhat positively chemotactic in molecular concentrations of 2 per cent and over. In lower concentrations it was neutral. This held true for the blood of man, rabbits, and dogs.

Sodium phosphate was positively chemotactic in varying degrees as the tri-, di-, and monobasic salt.

In the work on sodium phosphate, advantage was taken of the fact that pneumonia patients who had received large doses of sodium phosphate either by mouth or intravenously showed an increased number of leucocytes migrating into the agar containing sodium phosphate or calcium chloride. A normal person was used as control and the blood tested at intervals after the same doses of sodium phosphate. It was found that, alike in pneumonia and in health, many more cells migrated into agar containing  $\text{Na}_2\text{HPO}_4$  or  $\text{CaCl}_2$  than in the case of those people who had not received the sodium phosphate medication. A typical set of figures is shown below for the blood of patients who had received large doses of sodium phosphate, when tested with  $\text{Na}_2\text{HPO}_4$  agar.

$\text{Na}_2\text{HPO}_4$ .

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
Pneumonia blood.								
6,182	7,023	7,147	10,862	7,046	7,136	7,563	7,014	5,409
11,427	15,356	15,876	18,973	14,899	14,992	7,642	7,924	5,311
Normal human blood.								
4,847	4,939	4,883	4,439	4,872	4,976	4,895	3,869	2,197
6,436	6,854	6,973	9,037	6,853	6,693	3,246	3,386	2,201

The blood of all animals showed a positive chemotaxis with  $\text{Na}_3\text{PO}_4$  in molecular concentrations of 0.1 to 2 per cent; in concentrations

over 2 per cent there was a slight negative chemotaxis, probably due to the alkaline reaction.

With both  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  marked positive chemotaxis was produced, as shown by the two following tables:

*$\text{NaH}_2\text{PO}_4$  with Normal Human Blood.*

4 per cent.	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	Control.
4,387	4,097	4,028	4,538	4,538	3,327	3,107	2,145	2,047
4,038	6,359	6,109	7,537	7,634	6,094	6,024	2,214	2,046

*$\text{Na}_2\text{HPO}_4$  with Normal Human Blood.*

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	Control.
10,847	5,432	7,096	7,756	4,483	3,224	2,875	2,846
9,856	8,951	9,943	8,926	6,453	4,954	4,493	2,893

When to calcium chloride  $\text{NaH}_2\text{PO}_4$  was added, there was a marked increase in the number of cells adhering. There seemed to be a synergistic action between sodium phosphate and calcium chloride, the increase in the number of cells being greater than could be accounted for by the addition of the cells adhering with either of the two chemicals alone.

*$\text{CaCl}_2$  Plus  $\text{NaH}_2\text{PO}_4$ .*

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
10,752	11,765	10,967	9,870	10,236	9,846	3,245	2,346
13,257	14,735	11,364	12,453	13,247	9,364	6,325	2,433

*Potassium Salts.*

All potassium salts were negatively chemotactic for all blood of all animals employed when concentrations over 0.01 per cent (molecular concentration) were used. This held true for potassium chloride, potassium acid carbonate, and potassium acid phosphate. In molecular concentration below 0.01 per cent there was a neutral response with all blood, human, rabbit, dog, and guinea pig.

*Barium Salts.*

Barium sulfide was slightly negatively chemotactic in molecular concentration over 0.05 per cent. Barium sulfate was slightly positively chemotactic.

*Strontium Chloride.*

Strontium chloride was slightly positively chemotactic, but strontium chloride with equal molecular concentrations of magnesium sulfate gave a great increase in the number of cells adhering to the agar. Here again the action seemed synergistic, since neither alone gave marked reaction, and many more cells adhered to the agar than could be accounted for by mere addition of the cells emerging when both salts were used.

*Magnesium Salts.*

Magnesium salts acted in the same way as sodium salts in regard to chemotaxis, this seeming to depend largely on the negative ion attached to the magnesium. Magnesium sulfate was neutral as regards chemotaxis in all concentrations for all blood. Magnesium tartrate was slightly negatively chemotactic in concentrations over 1 per cent, but neutral in all concentrations below this for all blood used. Magnesium citrate was slightly negatively chemotactic for all blood but more so for rabbit than for human blood.

*Mercury Salts.*

All mercury salts behaved about the same in regard to chemotaxis. They seemed to fix the blood before the cells could migrate into the agar, so that only a few could be counted. The clot formed was hard and brittle. This apparent negative chemotaxis of mercury salts is really a fixation of proteins and not a chemotaxis of any kind. For these reasons, the action of these compounds must be studied in some other way to determine the degree of chemotaxis they possess.

*Organic Acids.*

Oleic acid was positively chemotactic. This substance was used with the paraffin method so that the acid would dissolve quickly

throughout. Since, however, only the sides of the cell thus formed contained the reagent, this was unsatisfactory. For this reason, an emulsion of oleic acid with agar was made and then cooled and hardened rapidly over ice so that the floor and sides of the cell thus formed would contain the same concentration of the reagent. There was an increase of 100 per cent in the number of cells adhering at 0.5 per cent concentration over the control; in concentrations over 1 per cent there was no chemotactic effect, about the same number of cells adhering as in the control. The sodium salt of this acid showed only a very slight increase in the number of cells adhering at 0.1 per cent molecular concentration; above this there was a slightly negative chemotaxis.

Lactic acid was negatively chemotactic. This may have been due in part to the hydrogen ion concentration, but the sodium salt was also negatively chemotactic although not so markedly so as the pure acid. There were only half as many cells adhering with lactic acid in concentrations above 0.05 per cent as there were in the control.

Uric acid was slightly negatively chemotactic in molecular concentrations above 0.5 per cent; below this concentration it had no effect.

Nucleinic acid made from yeast was positively chemotactic in molecular concentrations of 0.0005 to 0.00001 per cent or percentage concentrations of 0.0065 to 0.00013; when the acidity was overcome by the addition of a slight amount of sodium hydroxide (hydrogen ion concentration  $\text{pH}=7.7$ ), there was an increase in the number of cells adhering. With the free acid there were two-thirds more cells adhering at 0.0025 per cent molecular concentration (0.0325 per cent by weight) than in the control. With the addition of the sodium hydroxide there were twice as many cells adhering as in the control.

#### *Amino-Acids and Amines.*

Glycine and alanine were slightly positively chemotactic, alanine being somewhat more so than glycine. At 4 per cent concentration there were about three times as many cells adhering as in the control when glycine was used, while with alanine there were about three and one-half times as many cells adhering.



Tyrosine was somewhat positively chemotactic. There were about three times as many cells adhering with molecular concentrations of 0.0001 to 0.001 per cent as in the control. Tyramine, made synthetically,<sup>6</sup> acted most peculiarly. The cells clumped in such a way that it was impossible to count or even to estimate the number migrating into the agar with any degree of exactitude. This was repeated many times, the same thing occurring each time. No reason could be found for this action of tyramine. It suggested the appearance of a calcium cyanide clot, but differed in that the cells were much more firmly adherent to each other with tyramine than with calcium cyanide.

Histamine<sup>7</sup> was strongly positively chemotactic in molecular concentrations of over 0.000025 per cent (0.000055 per cent by weight), but the leucocytes of dog and human blood showed a greater degree of migration into the agar containing histamine than did the leucocytes of rabbit blood. One set of illustrative figures will be given for human, one for dog, and one for rabbit blood.

*Histamine.*

0.005 per cent.	0.00025 per cent.	0.0001 per cent.	0.00005 per cent.	0.000025 per cent.	0.00001 per cent.	0.000005 per cent.	0.0000025 per cent.	Control.
Human blood.								
7,021	6,538	5,028	2,537	3,159	1,138	1,274	1,485	1,407
7,183	6,983	9,894	1,093	4,963	2,915	1,019	1,857	1,396
Dog blood.								
5,379	5,027	2,473	1,532	2,036	1,463	3,527	4,037	3,016
7,037	7,239	7,635	6,528	6,017	3,012	5,172	5,187	3,423
Rabbit blood.								
4,507	4,017	3,012	1,093	1,004	1,025	1,028	857	1,046
—	4,321	3,294	993	3,653	1,946	1,956	1,845	1,243

Histidine from the same source showed a slight positive chemotaxis for all blood, human, dog, and rabbit. There was an increase of about

<sup>6</sup> The tyramine was made by Dr. Koessler and Dr. Hanke.

<sup>7</sup> This was also obtained from Dr. Koessler and Dr. Hanke.

25 per cent in the number of cells adhering at 0.005 per cent over the number adhering in the control window.

Glutamic acid was slightly positively chemotactic in concentrations of 0.0005 to 0.00001 per cent; when the acidity was overcome by the addition of a slight amount of sodium hydroxide, the solution being neutral to phenolphthalein ( $\text{pH}=7.7$ ), there was an increase in the number of cells adhering. When the free acid was used there was an increase of 100 per cent in the number of cells adhering with concentrations of 0.005 to 0.00001 per cent, while with the addition of sodium hydroxide the increase was 200 per cent over the control.

Aminovalerianic acid was positively chemotactic in all concentrations to about the same extent as glutamic acid. With the addition of sodium hydroxide there was also an increase in the number of cells adhering. A typical series for aminovalerianic acid and human blood is:

*Aminovalerianic Acid.*

0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	0.0001 per cent.	0.00005 per cent.	0.00001 per cent.	0.000005 per cent.	Control.
3,537	4,091	4,486	3,147	2,159	2,038	1,537	2,046	3,034	1,986
6,283	5,897	5,546	4,138	5,589	3,143	3,107	3,186	3,037	2,037

Aminovalerianic acid with NaOH added to neutrality.

4,031	4,899	4,998	3,887	3,926	3,967	4,037	3,448	3,963	2,015
9,837	9,853	9,475	6,015	4,084	3,996	3,026	4,035	5,863	2,104

*Alkaloids.*

Morphine was slightly positively chemotactic for human and dog blood in most concentration for all morphine compounds used except the citrate. This is contrary to most of the work that has been done with morphine and its compounds, but after many repetitions the same result was obtained. There is much less increased migration of leucocytes with rabbit than with human blood. Morphine sulfate showed a slight but constant positive chemotaxis for human and dog blood in concentrations of 0.3 to 2 per cent, for rabbit blood in concentrations of 3 per cent. Morphine, morphine hydrochloride and nitrate,

all showed the same degree of chemotaxis. Morphine citrate was practically neutral in concentrations below 0.05 per cent, but above that it was negatively chemotactic.

*Morphine Hydrochloride.*

3 per cent.	2 percent.	1 percent.	0.5 percent.	0.1 percent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
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Human blood.

1,817	1,537	1,509	1,598	1,648	1,892	2,147	3,027	2,568	1,785
2,047	5,538	5,018	5,537	5,536	5,023	4,321	4,572	4,012	1,825

Rabbit blood.

3,018	1,016	1,546	1,512	1,478	1,443	1,976	2,314	2,215	2,415	2,648
4,001	1,192	2,032	1,472	2,351	2,511	1,897	1,998	2,322	2,451	2,771

Heroine and heroine compounds were neutral as regards chemotaxis. Codeine and codeine compounds were neutral in most concentrations, but in concentrations over 1 per cent there was a slight negative chemotaxis.

Caffeine sulfate was negatively chemotactic for all blood in all concentrations. Quinine sulfate, strychnine sulfate, and brucine sulfate were all negatively chemotactic for all blood in all concentrations.

*Miscellaneous Substances.*

Papayotin, or papain, was markedly negatively chemotactic for human blood in all concentrations. In fact, only 5 to 100 leucocytes adhered to the agar containing concentrations of papayotin over 0.05 per cent. With rabbit blood there was a remarkable difference, a positive chemotaxis being observed with all concentrations over 0.05 per cent. This decided chemotaxis for rabbit blood is mentioned by Gabritchevsky. Dog blood came midway between rabbit and human blood, while the blood of frogs varied with the temperature of the body; thus the winter frog blood was negatively chemotactic, the summer frog blood was positively chemotactic.

*Papayotin.*

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	Control.
Human blood.								
120	110	100	102	135	385	732	983	2,361
16	34	35	74	52	87	128	1,184	2,752
Rabbit blood.								
6,284	6,938	6,733	5,732	3,462	3,352	2,573	2,283	4,198
6,988	6,793	6,990	5,938	4,521	3,710	3,109	2,846	4,017

Urea, urethane, and creatinine were all negatively chemotactic for the blood of all animals, in molecular concentrations above 0.5 per cent; below this concentration they were neutral.

Ethyl alcohol was negatively chemotactic in all concentrations over 2 per cent, no matter whether the paraffin or agar method was used. In concentrations under 2 per cent, alcohol was neutral.

Cantharidin, or cantharidinum (U.S.P.), was positively chemotactic. Cantharidin was obtained from Chinese cantharides and the purified cantharidin was dissolved in 70 per cent alcohol and an emulsion made with agar. This emulsion was cooled rapidly over ice, then the windows were cut as in previous cases. The blood of different animals varied markedly towards cantharides. This was strikingly shown for rabbit blood. The rabbit seems to have a congenital tolerance for cantharidin, and rabbit leucocytes were little influenced by cantharidin, which was decidedly positively chemotactic for leucocytes from other species.

*Cantharidin.*

0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
Human blood.									
5,987	3,863	3,993	7,946	5,124	2,136	1,872	2,044	2,115	2,109
1,928	6,937	4,837	8,123	5,923	1,927	1,764	2,942	2,012	2,213
Rabbit blood.									
6,001	4,336	4,118	2,119	2,139	2,003	1,947	3,024	3,642	3,798
1,125	2,453	1,362	2,774	3,632	3,587	1,176	3,531	3,346	3,631

It will be noticed that when the concentration of cantharidin was over 0.25 per cent, fewer cells migrated into the agar combined with cantharidin than into the plain agar with a margin of agar plus cantharidin. It is possible that strong concentrations of markedly chemotactic substances have a repelling action. This is shown more markedly in the case of rabbit blood, for here there seems to be a repelling action in all concentrations over 0.05 per cent in the cells with a cantharidin-containing floor.

Aspirin (acetyl-salicylic acid), acetanilide, and chloral hydrate were all negatively chemotactic in all concentrations for all blood. The reduction in the number of cells adhering varied from 20 to 60 per cent below the normal. Fewer cells adhered in concentrations over 0.5 per cent than below. At 4 per cent concentration, with all of these reagents, only about 40 per cent of the control number of leucocytes adhered.

Chloretone (Parke, Davis and Company) was slightly positively chemotactic in practically all concentrations, the increase varying from about 5 to 20 per cent over the control.

Glucose was neutral in all concentrations under 20 per cent; above that there was a very slight negative chemotaxis.

Mustard gas (dichloroethyl sulfide) was practically neutral in all concentrations. An emulsion was made of the sulfide in oil with agar and then cooled rapidly over ice. When the sulfide in oil was added to paraffin, more of the gas volatilized because the oil had to be added at a much higher temperature, due to the higher melting point of the paraffin than of the agar. With paraffin, also, the floor consisted of the plain glass so that the effects of the reagent could be seen only along the sides of the window thus made.

Parazol, or crude dinitrodichlorobenzene,<sup>8</sup> was somewhat positively chemotactic in concentrations under 1 per cent; over that it was negatively chemotactic.

Olive oil was neutral in all concentrations.

Scarlet R, purified, was slightly positively chemotactic in concentrations of 0.5 to 2 per cent, below that it was neutral.

<sup>8</sup> Concerning the toxicity and properties of parazol see Wells, H. G., *J. Ind. Hyg.*, 1920, ii, 247; Voegtlin, C., Livingston, A. E., and Hooper, C. W., *Bull. Hyg. Lab., U. S. P. H., No. 126*, 1920, 183.

Turpentine was slightly positively chemotactic in concentrations over 0.1 per cent; there was an increase of 50 per cent over the control at 0.5 per cent concentration.

Veronal was slightly negatively chemotactic in all concentrations over 0.5 per cent; below that it was about neutral.

Yeast vitamine (Harris) was slightly positively chemotactic in concentrations over 0.1 per cent. This may be due to the fact that many amino-acids and extractives are contained in this substance, and as they are positively chemotactic, anything in which they are found will show a certain degree of positive chemotaxis.

Ether was slightly positively chemotactic in concentrations over 0.1 per cent, when there was an increase of about 50 per cent over the control in the number of cells adhering.

#### DISCUSSION.

This new method of Wright's has been found very satisfactory for the determination of chemotaxis *in vitro*. It has been modified somewhat; *i.e.*, there is no need for calibrated capillary pipettes, as ordinary capillary pipettes may be used and 1 or 2 drops from the same pipette will give the same amount of blood. Cooling will cause changes in the reaction, so that it is necessary to keep the blood at 37°C. at all times. The hydrogen ion concentration is an important factor and this has been considered carefully.

This method is definitely quantitative. All steps may be carefully controlled, so that factors of error may be practically eliminated. Substances may be used with this method that have heretofore never been studied as regards their chemotactic powers; among these are cantharidin, histamine, mustard gas, parazol, and tyramine. The results obtained by this method with substances frequently studied, agree in practically all particulars with those obtained by other workers with different methods. It may also be employed as a method of determining the action of unknown drugs in producing the migration of leucocytes at the site of injection. The results obtained with sodium phosphate compounds, showing them to be strongly positively chemotactic and increasing the chemotaxis of the blood of those taking them either by mouth or intravenously, are

of interest in relation to recent work on the use of these salts in the treatment of many infections.

It has been found that the blood of different species of animals may react differently towards different drugs; thus, rabbit blood shows much less positive chemotaxis for cantharidin than human or dog blood. On the other hand, rabbit blood was much more markedly positively chemotactic for papayotin than human or dog blood. With histamine, human and dog blood showed more cells migrating into the agar than did rabbit blood. There seems to be no apparent underlying reason for this difference in action of the blood of different animals.

#### CONCLUSIONS.

1. Wright's method for the study of chemotaxis of leucocytes *in vitro*, slightly modified, has been found to be most satisfactory in the estimation of the degree of chemotaxis of various substances, because it is possible to make an exact quantitative determination of the leucocytes that have migrated from the blood clot and adhere to the surfaces containing the tested substance.

2. The calcium ion is the only inorganic ion *per se* which is found to be positively chemotactic under the conditions of these experiments. It is markedly chemotactic in all concentrations and in all combinations, except the citrate. Here the negative chemotaxis of the citrate ion neutralizes the positive chemotaxis of the calcium ion, and neutrality of chemotactic effect results.

3. The sodium and magnesium ions themselves are neutral. Magnesium and sodium salts are dependent upon the negative ion with which the magnesium or sodium is combined for such positive or negative chemotaxis as is exhibited. All the phosphates of sodium, whether tri-, di-, or monobasic salts, are markedly positively chemotactic, and when combined with other reagents which are themselves neutral or negatively chemotactic, produce marked positive chemotaxis. The blood of a person who has taken phosphates either by mouth or intravenously shows a great increase in chemotaxis with sodium phosphate, with calcium chloride, and even with sodium chloride which is ordinarily neutral.

4. All potassium salts are negatively chemotactic.

5. Many substances act synergistically as regards chemotaxis; *e.g.*, when strontium and magnesium salts are mixed there is a marked increase in chemotaxis. Sodium phosphate acts synergistically with calcium chloride.

6. Mercury salts fix the leucocytes in this method so that their influence on chemotaxis cannot be determined.

7. Morphine and morphine salts are positively chemotactic; this is contrary to the results obtained by others with different methods.

8. Substances which produce a very acute inflammation, such as cantharidin, histamine, or turpentine, are found to be positively chemotactic by this method, but substances, such as mustard gas, which produce a marked necrotizing effect are found to be negatively chemotactic, or neutral, though physiologically they would appear to be positively chemotactic.

9. All amino-acids and amines are positively chemotactic to a certain extent. It seems that the longer the carbon chain, the greater the degree of chemotaxis, though this is not absolute. Tyramine is one exception to this, for it causes a peculiar clumping of the cells, so that it is impossible to count the number adhering, and thus determine whether or not tyramine is positively chemotactic.

10. The time that the blood of animals is examined after eating makes a marked difference in the number of cells adhering, for shortly after eating, within 30 minutes, very many more cells will adhere to the agar than at a later time.

11. The blood of different species of animals reacts differently towards different reagents. The chemical composition of these agents seems to have nothing to do with this difference in reaction as far as we could determine.

12. With frozen serial sections it has been found that the depth of penetration of the leucocytes into the agar is proportional to the positive chemotaxis produced by the substance combined with the agar, as demonstrated by the number of leucocytes adherent to the walls of the test chambers.



# WANDERING CELLS, ENDOTHELIAL CELLS, AND FIBROBLASTS IN CULTURES FROM HUMAN LYMPH NODES.

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PLATES 26 TO 32.

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In recent communications on the cultivation of human lymph nodes, lymphocytes and giant cells have been described.<sup>1, 2</sup> This paper, to complete the series, will be concerned with observations on wandering cells, reticulum cells, endothelial cells, and fibroblasts, with a discussion as to the significance of their morphological and physiological similarity.

Lymph glands, excised aseptically from patients in the Johns Hopkins Hospital, were brought to the laboratory and planted from  $\frac{1}{2}$  to 2 hours after their removal. They were cut into small bits in sterile Locke-Lewis solution, placed as usual upon a sterile No. 1 cover-slip, covered with a drop of homogenous or autogenous plasma, inverted over a hollow ground slide rimmed with vaseline, and immediately put into an incubator with a temperature of 37.5°C. Twenty to sixty cultures were prepared from each gland; this afforded an opportunity for a considerable number of studies lasting from 2 hours to 3 weeks.

The seven cases of normal glands showed relatively few of the cells under discussion, as did the cases of melanosarcoma, carcinoma, and lymphoid and myeloid leukemia. But in the two cases of sarcoma, the three cases of chronic lymphadenitis, two cases of Hodgkin's disease, and seven cases of tuberculous lymphadenitis, they were very

<sup>1</sup> Lewis, W. H., and Webster, L. T., *J. Exp. Med.*, 1921, xxxiii, 261.

<sup>2</sup> Lewis, W. H., and Webster, L. T., *J. Exp. Med.*, 1921, xxxiii, 349.

numerous, and in each case the relative and absolute quantity of cells seen in the cultures was proportional to that seen in the corresponding histological sections stained with hematoxylin and eosin.

### *Wandering Cells.*

Histological sections of normal and pathological human lymph glands show deeply staining mononuclear cells with abundant cytoplasm, scattered about the sinuses and through the lymph tissue. To this most common type of loose tissue cell, found as well in chronic inflammatory areas and foci of cellular infiltration, pathologists give the name wandering cell. Its origin will be referred to later.

After 2 or 3 hours incubation, wandering cells may be seen coming from cultures of normal glands, tuberculous glands, and glands with Hodgkin's disease and metastatic tumor. They follow the migrating lymphocytes which are first to leave the tissue fragment and form a ring about the explant between the cover-slip and plasma clot. They are irregular in size and shape but when flattened out on the cover-slip, they measure about 0.024 by 0.016 mm. They consist of a delicate clear zone of ectoplasm and an inner, slightly more opaque zone of endoplasm which contains mitochondria, fat globules, neutral red granules and vacuoles, and a single nucleus (Figs. 1 to 8 and 30).

The fat globules appear greenish and refractile; they take a Sharlach R stain readily. Although they vary in number, size, and distribution in cells at different ages, during the early hours of observation they are generally few in number, regular in size, and scattered indiscriminately throughout the endoplasm.

A few neutral red granules and an occasional vacuole are seen, but no note concerning a definite grouping of granules about a centrosphere area was made at this time.

The nuclear material has a waxy appearance and occupies from one-third to one-half of the cell volume. With the cell spread out at rest, the nucleus tends toward an oval or bean shape, but when the cell is actively moving, it adapts itself to conditions determining movement by assuming all possible forms from bizarre to spindle and dumb-bell outlines.

The cells move rapidly and generally away from the explant, although they frequently change their direction or remain motionless for a considerable period of time. The clear ectoplasm, which seems to be fluid, pushes out in a fan-shaped manner, advancing and retracting its pseudopods. Into this protrusion a few fat globules with active Brownian movement are seen to flow, followed by the nucleus. Finally the posterior part of the cell draws up and the crawling, flowing motion may temporarily cease. The average rate of migration is roughly estimated at 0.0306 mm. per minute. This is considerably faster than the migratory rate of lymphocytes (Fig. 14).

At 24 hours there is moderate migration of wandering cells in cultures from normal glands and glands with carcinoma metastases, while in cultures from chronic lymphadenitis, tuberculous lymphadenitis, and glands with sarcoma metastases and Hodgkin's disease, the wandering cells are present in great numbers. They are scattered radially about the explant and are most numerous in its vicinity.

Usually the cells show no alteration. The ectoplasm, still clear, contains rod-like and granular mitochondria, conspicuous when stained with Janus black No. 2. In the more inactive cells the ectoplasm tends to become irregular.

In the endoplasm fat globules and vacuoles are larger and more numerous. There are many neutral red granules, differing in size and shape. They may be scattered indefinitely throughout the cell or grouped at one side of the nucleus radially about a central clear area. Most of the cells are still active; nucleus and cytoplasm change shape and position rapidly.

These cells are active phagocytes. In the infected cultures, bacteria are crowded about the cell membrane and are contained in large numbers in the endoplasm. In other cultures hemoglobin pigment and disintegrating lymphocytes are engulfed by the wandering cells.

A few cells are greatly altered in appearance. They are much smaller and appear dark and shrivelled. The contracted ectoplasm shows many hair-like processes clinging to the cover-slip. The endoplasm appears as a shrunken, irregular, circular mass suspended from the under surface of the glass by the ectoplasmal processes. It

often contains one or two very large fat globules or vacuoles taking the neutral red stain. When visible, the nucleus is usually dark, sausage-shaped, and pushed to one side of the cell (Figs. 9 to 13).

At 48 hours an occasional wandering cell is seen spread out upon the cover-slip. The fat globules are larger and the neutral red area is composed of a rosette arrangement of granules at one side of the nucleus. Instead of active migration, movement is limited to changes in shape of the cell and relations of its component parts. By far the greater number are shrivelled and inactive, clinging to the cover-slip by spidery ectoplasmal processes.

At 5 days no active wandering cells are seen.

In brief, then, the wandering cell, found in the sinuses of lymph glands, about chronic inflammatory areas, and associated with so called round cell infiltrations, migrates from cultures of human lymph nodes. It appears in 2 or 3 hours, shortly after the lymphocytes, continues to move for about 48 hours, then gradually becomes quiescent, shrivels, and dies. Its structure and physiological characteristics make it easily recognizable.

#### *Endothelial Cells.*

In describing the histology of lymph nodes, text-books of Stöhr,<sup>3</sup> Schäfer,<sup>4</sup> and Szymonowicz<sup>5</sup> refer to lymphatic endothelium as lining capsule, trabeculae, lymph sinuses, and continuing as branched cells to form the general supporting framework, or reticulum. However, in our observations on migrating cells, we were unable to differentiate blood vessel endothelium, lymphatic endothelium, and reticular endothelium so the term endothelial cell will be used to designate a group of cells seen in cultures of normal and pathological lymph nodes morphologically and physiologically similar to each other and

<sup>3</sup> Stöhr, P., in Schultze, O., *Lehrbuch der Histologie und der mikroskopischen Anatomie des Menschen mit Einschluss der mikroskopischen Technik*, Jena, 16th edition, 1915, 141.

<sup>4</sup> Schäfer, E. A., *The essentials of histology*, Philadelphia and New York, 10th edition, 1916, 231.

<sup>5</sup> Szymonowicz, L., *Lehrbuch der Histologie und der mikroskopischen Anatomie mit besonderer Berücksichtigung des menschlichen Körpers einschliesslich der mikroskopischen Technik*, Würzburg, 3rd edition, 1915, 164.

to those seen in fixed preparations answering the above description. Endothelial cells migrate from explants of normal and pathological glands after 24 to 48 hours incubation. They are not active, so unless one chances to see a sudden streaming of granules and nucleus into advancing ectoplasm as the cell lengthens and momentarily changes its position, or unless by prolonged observation a rough average rate of 0.0002 mm. per minute is determined, the cells appear motionless. A predilection for clear areas between media or explant and cover-slip gives opportunity for careful study over considerable periods of time.

The typical endothelial cell, almost circular, measures about 0.04 mm. in diameter. One can distinguish clear ectoplasm with mitochondria and endoplasm containing nucleus, fat globules, mitochondria, and neutral red granules and vacuoles. The ectoplasm, when seen in its most characteristic form, appears as a broad circular band about three-fifths of the cell area whose outline, with irregular projections and indentations, changes somewhat from hour to hour. Scattered through the otherwise homogeneous ectoplasm are mitochondria in short threads or granules. Fat globules, limited to the endoplasm, vary in number, size, and distribution. Usually they are small and regularly scattered about the nucleus and centrosphere area. The neutral red granules form a rosette at one side of the nucleus. Small near the central clear area, they increase in size toward the periphery. About the neutral red area are mitochondria in short threads and granules. The nucleus is large, oval or bean-shaped, waxy in appearance, and motionless (Figs. 15 to 17).

Besides the so called characteristic forms, there are many variable cells tending on the one hand toward the smaller wandering cells and on the other hand toward the spindle or fibroblast cells. As these intermediate forms are studied, transitions may be seen, forming an unbroken series from wandering cells to endothelial cells and from endothelial cells to giant or spindle cells.

The transitions from endothelial cells to wandering cells are characterized by a decrease in the size of the cell and marked irregularity of outline. The circular shape gives way to irregular polyhedral and bizarre forms with ectoplasm seen chiefly as projections, hair-like threads, or long processes. Fat globules and neutral red granules

and vacuoles are more compact in the dense endoplasm, and the nucleus is smaller and less distinct. When these intermediate forms are actively ameboid or when they contain large numbers of disintegrating lymphocytes (Figs. 32 and 33) the cytological picture is, of course, modified, but in general the changes are as described above.

The transition forms from endothelial cells to spindle cells are more striking. The tendency to assume the bipolar shape is accompanied by an increase in surface area. Ectoplasm, very prominent, may flow out at one end and develop a long tail process at the opposite end while endoplasm and contents appear unchanged. Other cells show a triangularly shaped ectoplasm, some a dumb-bell elongation, and still others an outline resembling typical fibroblasts. No changes are observed in endoplasm or its constituent parts; the alteration is chiefly that of outline (Figs. 18 to 23, 28, and 29).

In the communication on giant cells<sup>2</sup> note was made of transition forms from large wandering cells or endothelial cells to giant cells with two, three, and four or more nuclei (Figs. 24 to 26).

12 to 24 hour observations on single endothelial cells determined their slow rate of motion and the infrequency of amitotic nuclear division and also brought to light actual changes in shape of the cells. Shrinking was observed with developing spicules and blebs; sudden acceleration of motion with a bipolar lengthening of the cell and a rapid flowing of nucleus and granules was not uncommon. The cell would move a short distance and then reassume its rounded form.

After 3 or 4 days incubation many cultures show a predominance of large cells of the endothelial type whose ectoplasm is either spread out irregularly upon the cover-slip or is branching and connected with adjacent cells. Here, all transitions from endothelial cells to fibroblasts may be seen; many branching cells contain two or three nuclei; typical giant cells are frequent (Fig. 31). It is possible that these may be true reticulum cells (Fig. 27).

At the end of the 1st week, lymphocytes and wandering cells have practically disappeared, leaving endothelial cells, giant cells, and transitional cells scattered through a network of fibroblasts (Fig. 34). Some of the endothelial cells shrivel, others disintegrate, so that after 16 days, very few remain.

In brief, then, endothelial cells migrate from explants of normal and pathological lymph glands after 24 to 48 hours incubation. They are large, almost without motion, constant in their cytological characteristics but are accompanied by related forms which simulate wandering cells or fibroblasts. The former may be active or phagocytic, the latter may show branched processes connecting similar cells.

### *Fibroblasts.*

Fibroblasts occur in the capsules and trabeculae of normal lymph glands and in scar tissue of many pathological glands. They appear in cultures from normal and pathological lymph nodes after 48 to 72 hours incubation. At this time the cells are usually spindle-shaped and migrate between cover-slip and plasma with their long axes parallel to radii from the explant. After the 4th or 5th day they may be branched and form a loose reticulum or network about the tissue fragment. They are often seen at this time in cultures which are otherwise quite devoid of growth.

Cytologically, they are similar to the fibroblasts of chick embryos described by Lewis.<sup>6</sup> The ectoplasm is usually bipolar and clear except for a few thread-like or granular mitochondria. Fat globules vary in number, size, and distribution. Neutral red granules and vacuoles may be grouped about a definite centrosphere area or may be scattered through the endoplasm. The nucleus is waxy in appearance and oval or bean-shaped (Fig. 35).

After 18 or 20 days, fibroblasts are still present and vigorous in many cultures.

### DISCUSSION.

In spite of Maximoff's recent work describing transition forms between lymphocytes and reticulum cells,<sup>7</sup> we have been unable to demonstrate any such relationship. However, we are impressed with the similarity of wandering cells to endothelial cells. Morphologically, differences are of apparent size and outline while the quality of nucleus, endoplasm and contents, and ectoplasm is the

<sup>6</sup> Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

<sup>7</sup> Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 222.

same. Physiological variations concern degree rather than nature of activity. Prolonged observations of single endothelial cells demonstrate short periods of activity accompanied by changes in shape. The frequent series of transitional forms lend further support to the theory of Marchand<sup>8</sup> that the wandering cells of lymph glands arise from the endothelial cells.

The large branching cells, appearing after the 3rd or 4th day, described with the endothelial cells, may well be true reticulum cells.

#### CONCLUSIONS.

Wandering cells migrate from cultures of human lymph nodes after 2 or 3 hours incubation; they are actively ameboid and phagocytic.

Endothelial cells appear in the plasma clot after 24 to 48 hours incubation. They are relatively inactive and less phagocytic.

Fibroblasts are seen after 48 hours incubation. They are inactive and not phagocytic.

The probable origin of wandering cells from endothelial cells is discussed.

#### EXPLANATION OF PLATES.

##### PLATE 26.

FIGS. 1 to 13. Wandering cells from cases of tuberculous lymphadenitis (Series 18). Fixed after 21 hours incubation.  $\times 750$ .

##### PLATE 27.

FIG. 14. Normal gland (Series 13). 4 hours incubation at 37.5°C. A wandering cell drawn at 1 minute intervals. Arrow points to explant.  $\times 559$ .

##### PLATE 28.

FIGS. 15 to 17. Typical endothelial cells from cases of tuberculous lymphadenitis (Series 18-9). Fixed after 2 days incubation.  $\times 750$ .

FIGS. 18 to 23. Transitional forms from endothelial cells to spindle cells from cases of sarcoma and tuberculous lymphadenitis (Series 8-9). 3 and 5 day cultures.  $\times 750$ .

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<sup>8</sup> Marchand, F., *Verhandl. deutsch. path. Ges.*, 1913, xvi, 5.



## PLATE 29.

FIGS. 24 to 27. Giant cells from a case of tuberculous lymphadenitis (Series 9). 5 day culture.  $\times 750$ .

FIGS. 28 and 29. Branching transitional endothelial cells from a case of tuberculous lymphadenitis (Series 9). 5 day culture.  $\times 750$ .

## PLATE 30.

FIG. 30. Wandering cells from a case of tuberculous lymphadenitis (Series 9). Fixed after 21 hours incubation.

FIG. 31. Endothelial cells and giant cells from a case of tuberculous lymphadenitis (Series 9). Fixed after 4 days incubation.

## PLATE 31.

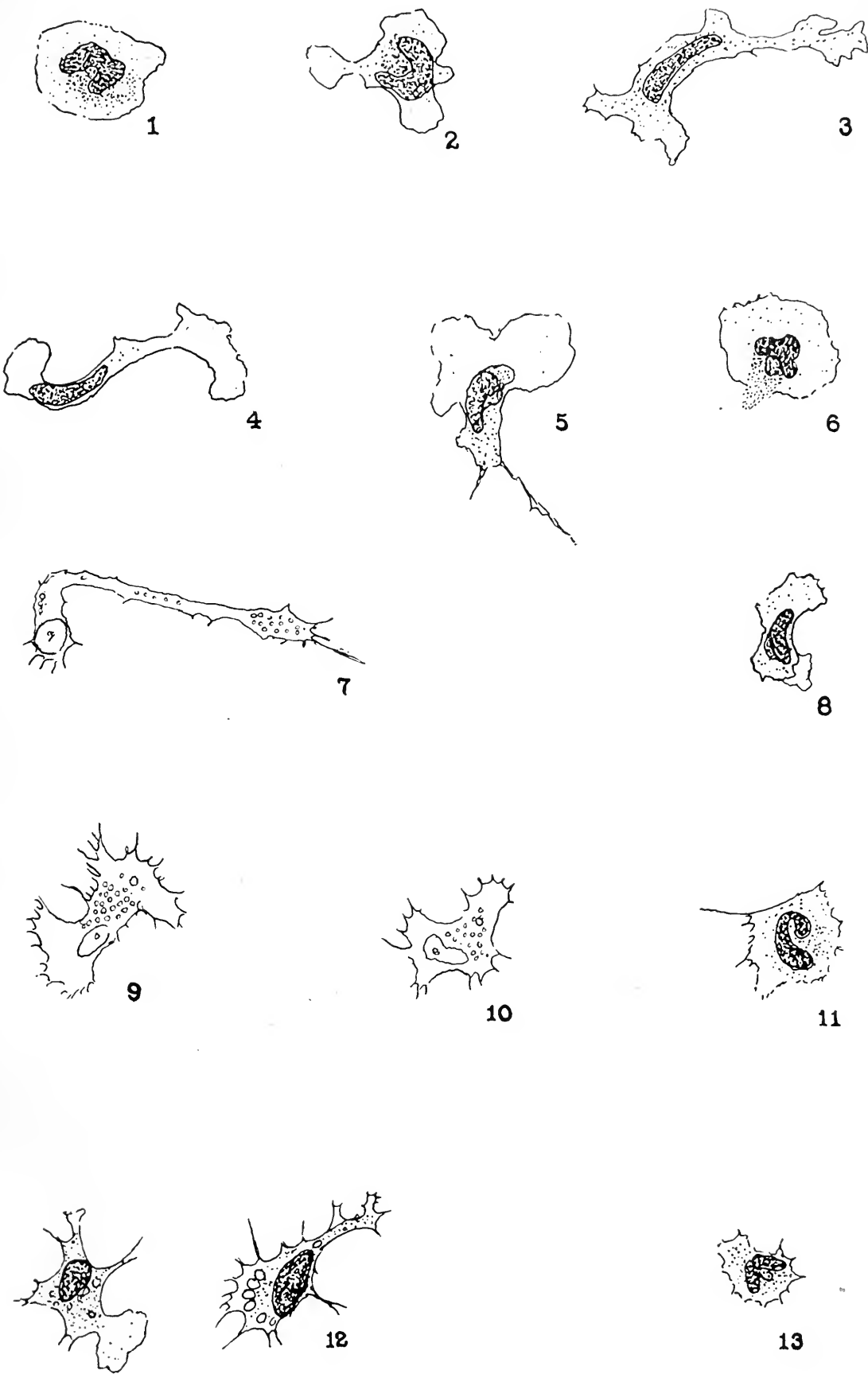
FIGS. 32 and 33. Phagocytosis by wandering cells and endothelial cells. Fig. 32. Tuberculous lymphadenitis (Series 7). 4 day culture. Fig. 33. Tuberculous lymphadenitis (Series 18). Fixed after 2 days incubation.

## PLATE 32.

FIG. 34. Endothelial cells, transitional cells, and spindle cells from a case of tuberculous lymphadenitis (Series 18). 7 day culture. Fixed.

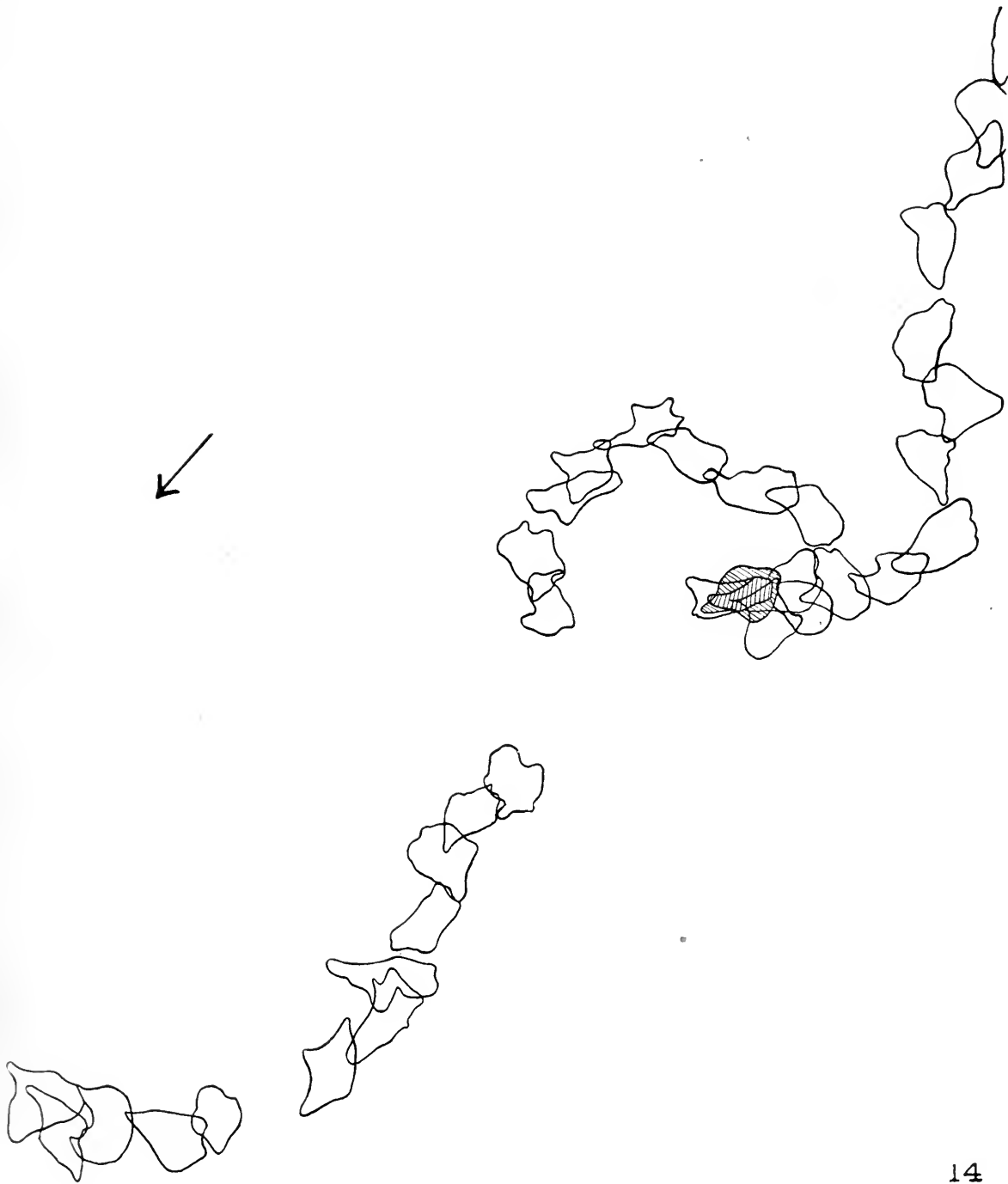
FIG. 35. Spindle cells from a case of tuberculous lymphadenitis (Series 9). 9 day culture. Fixed.

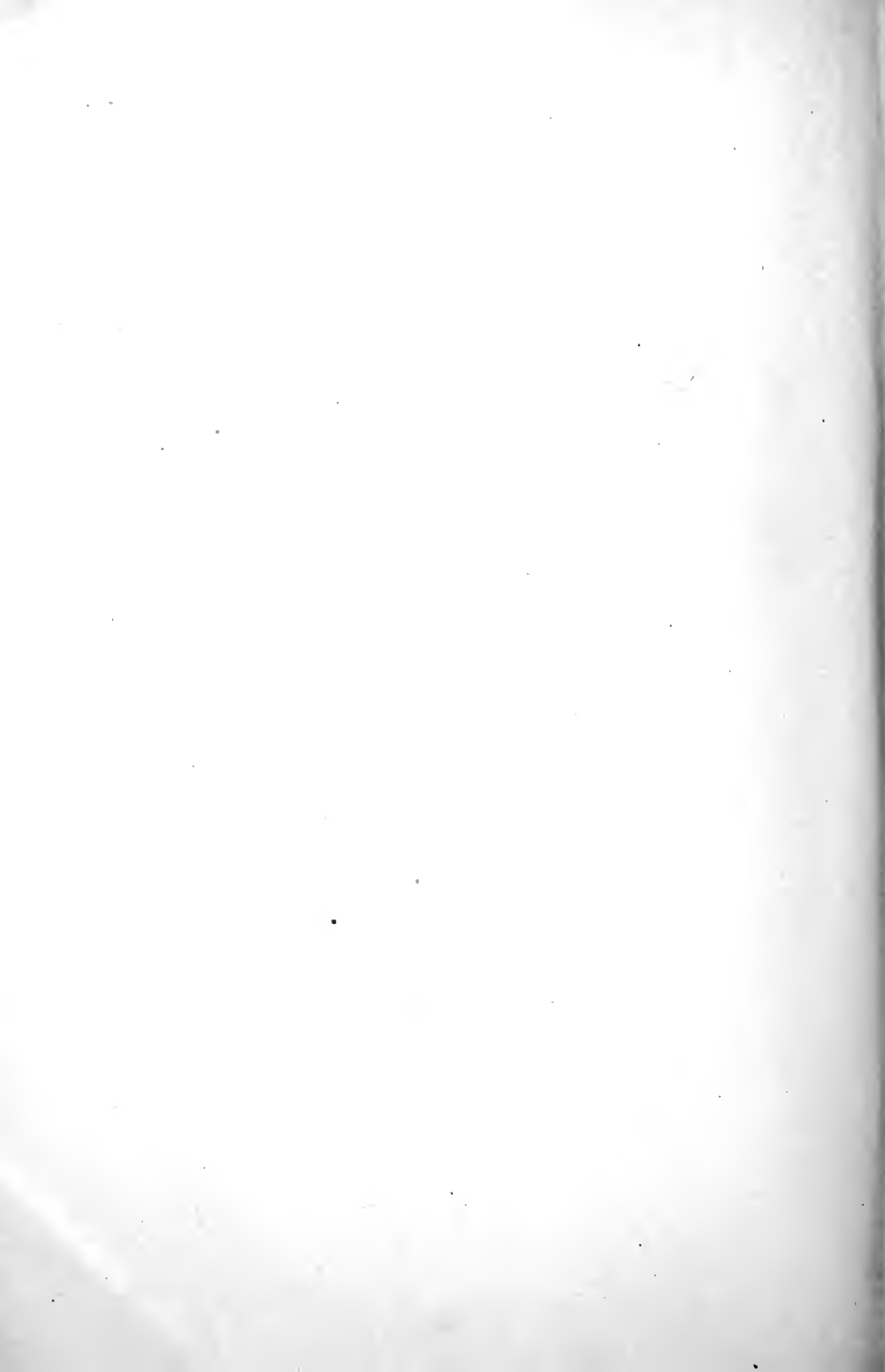
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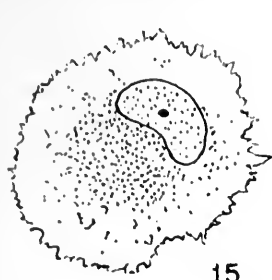


(Lewis and Webster: Cultures from human lymph nodes.)

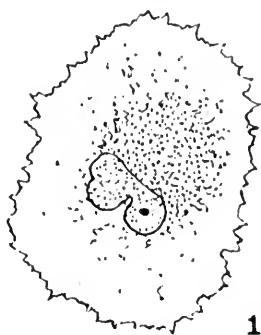




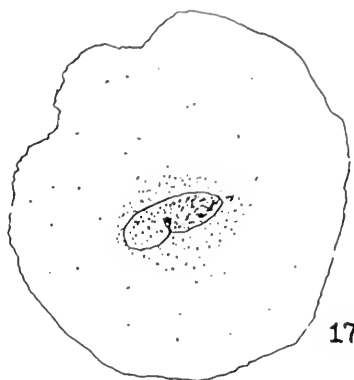




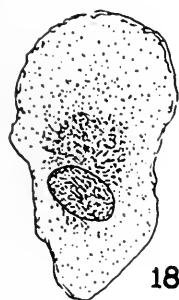
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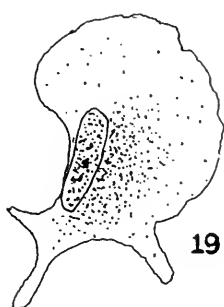
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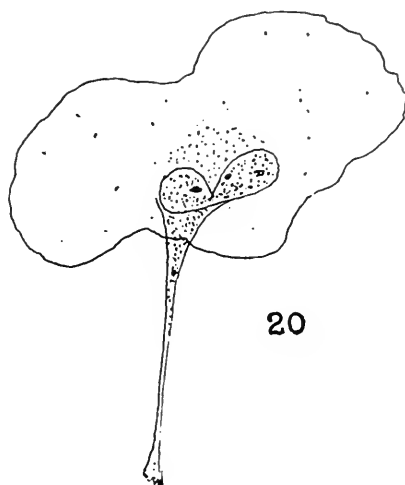
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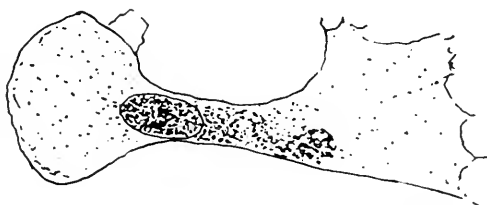
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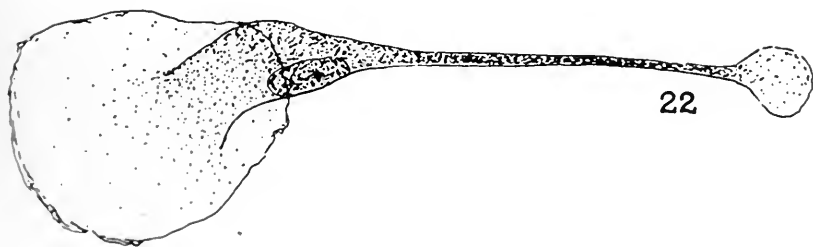
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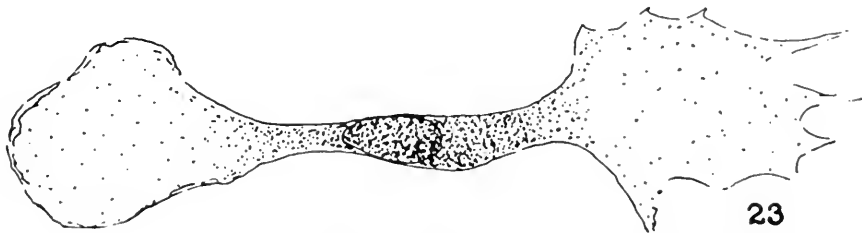
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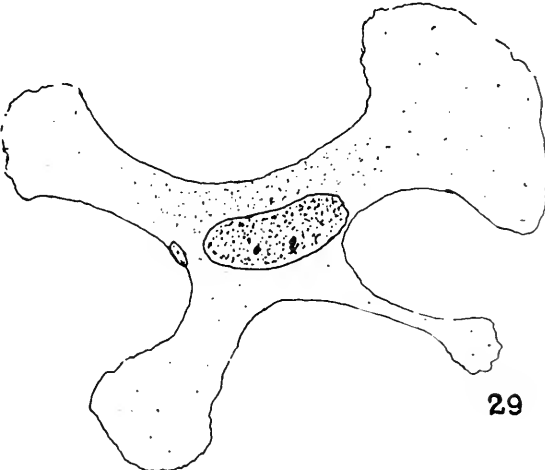
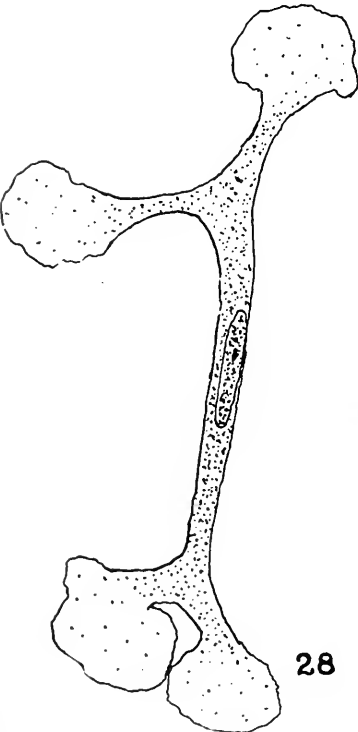
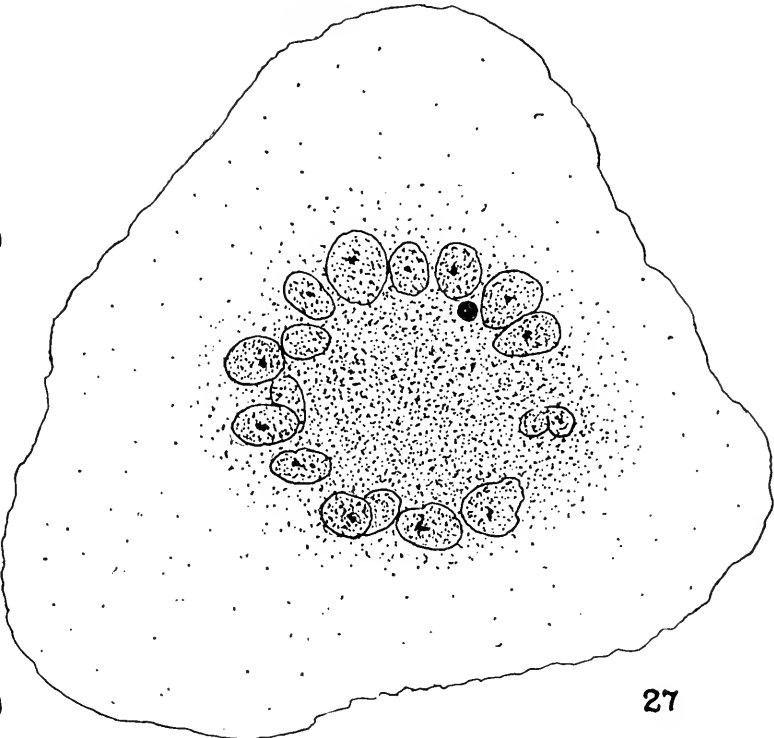
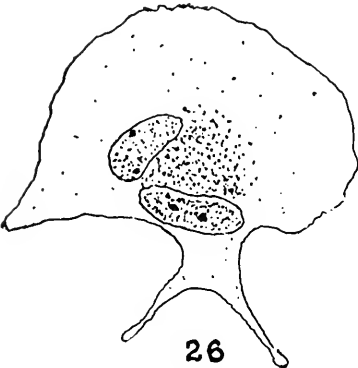
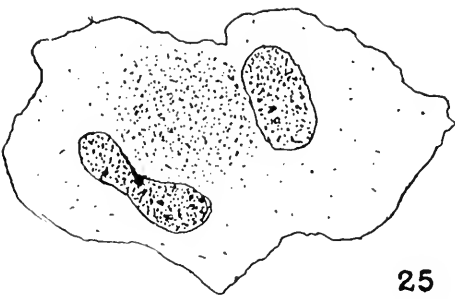
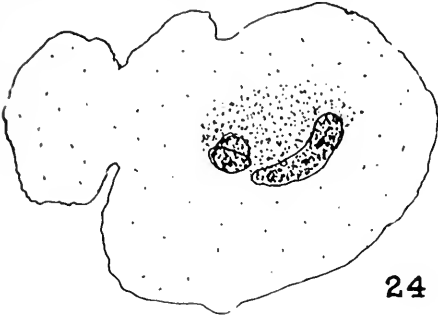


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(Lewis and Webster: Cultures from human lymph nodes.)

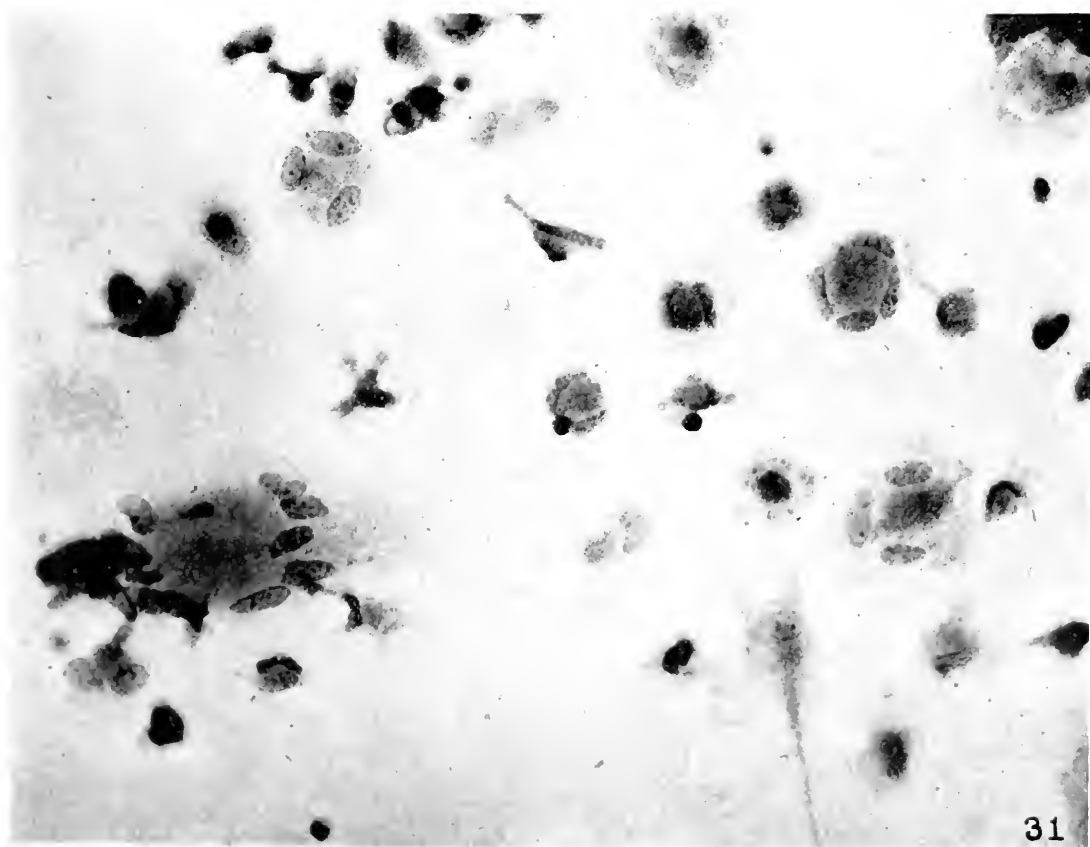
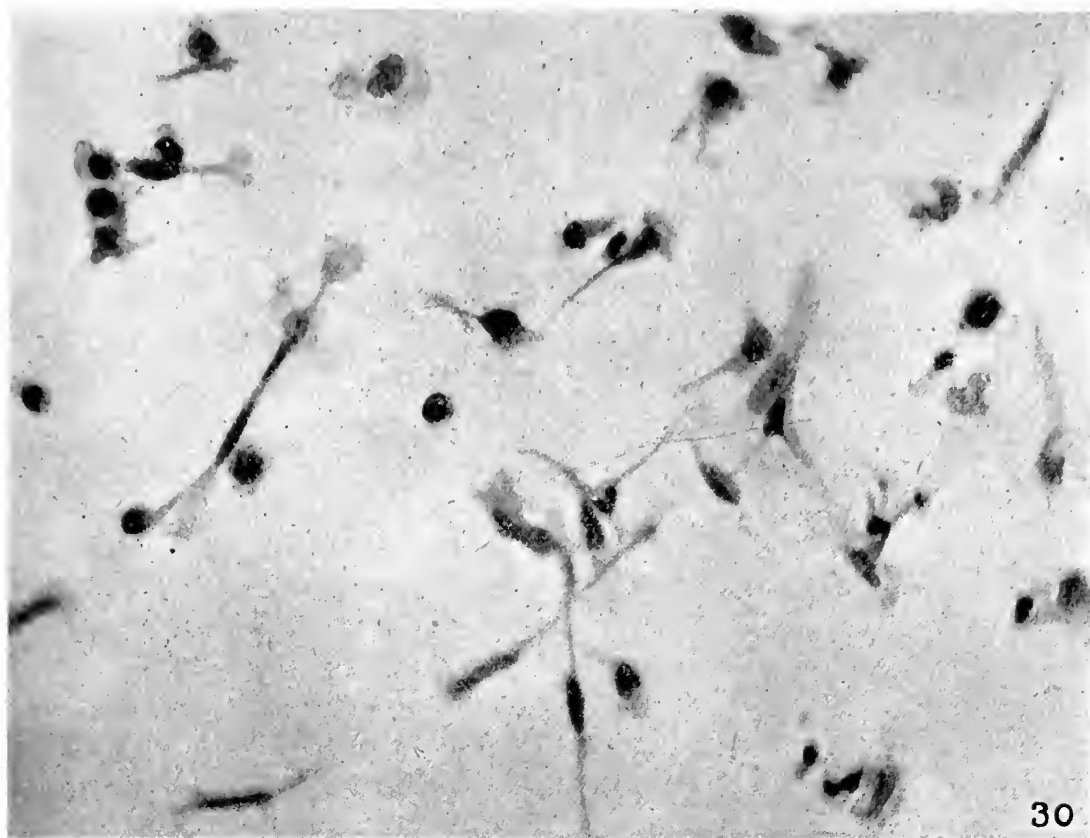






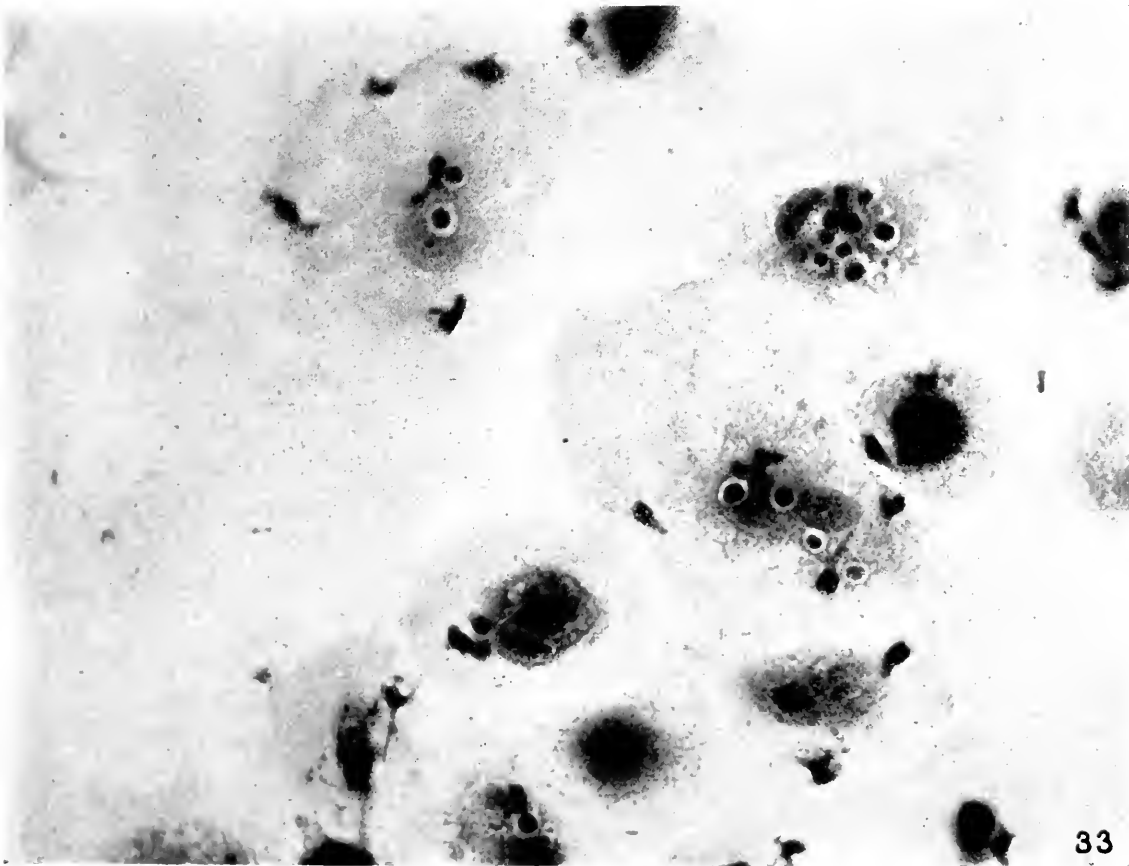
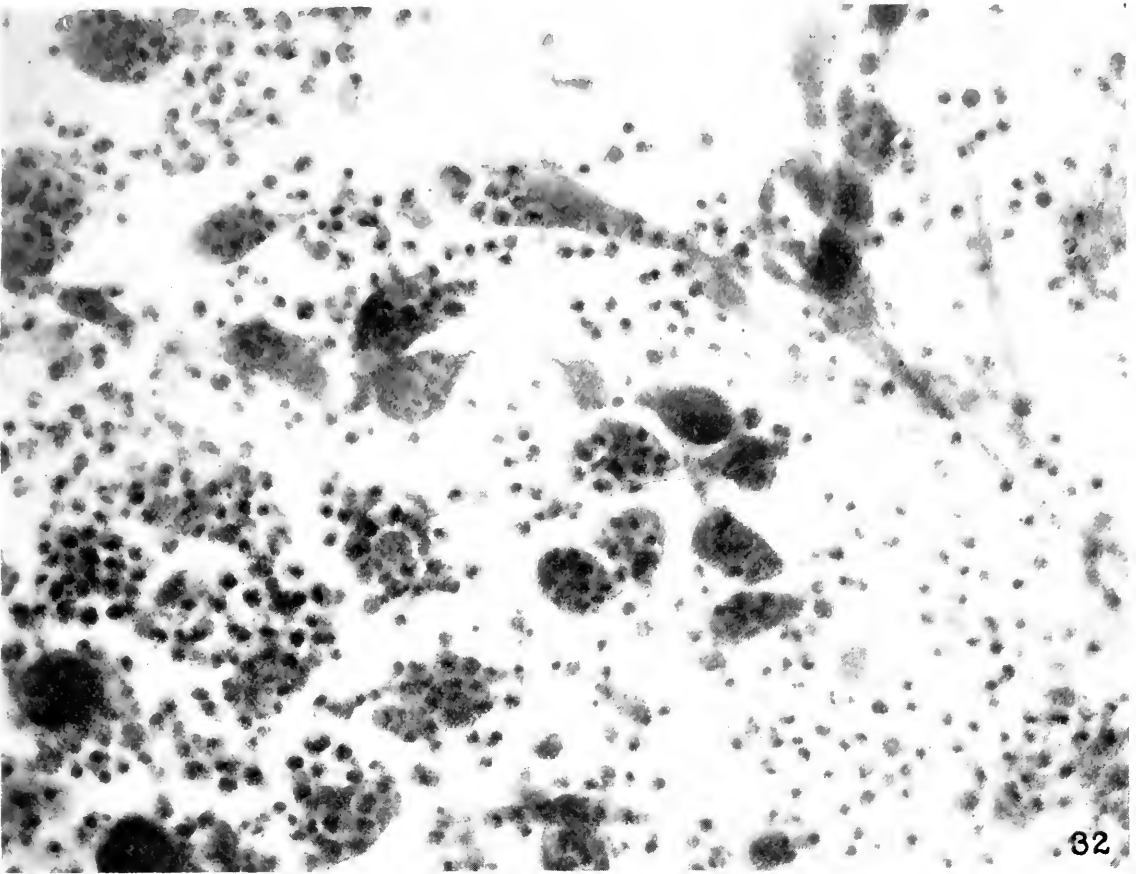
(Lewis and Webster: Cultures from human lymph nodes.)



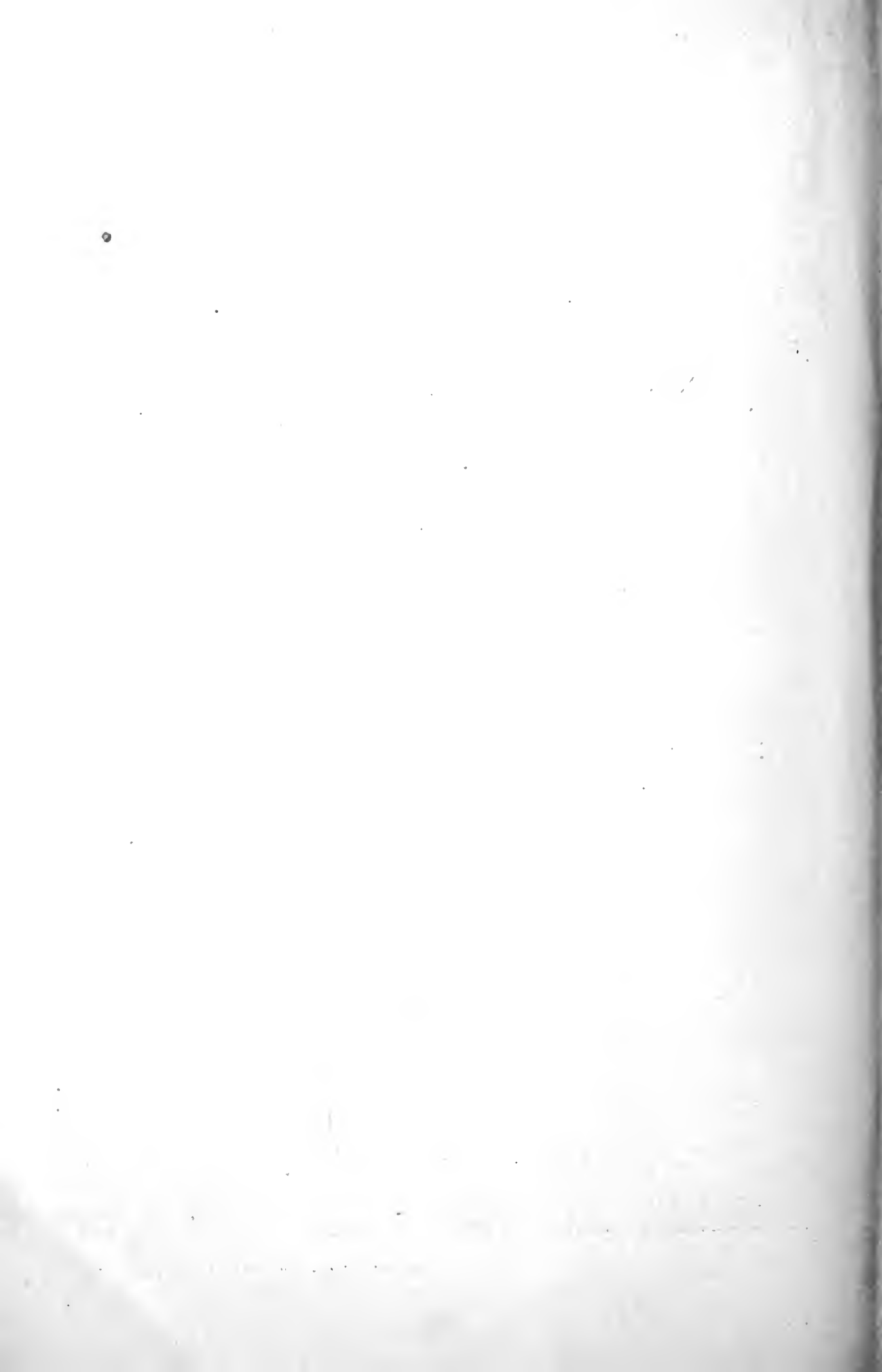


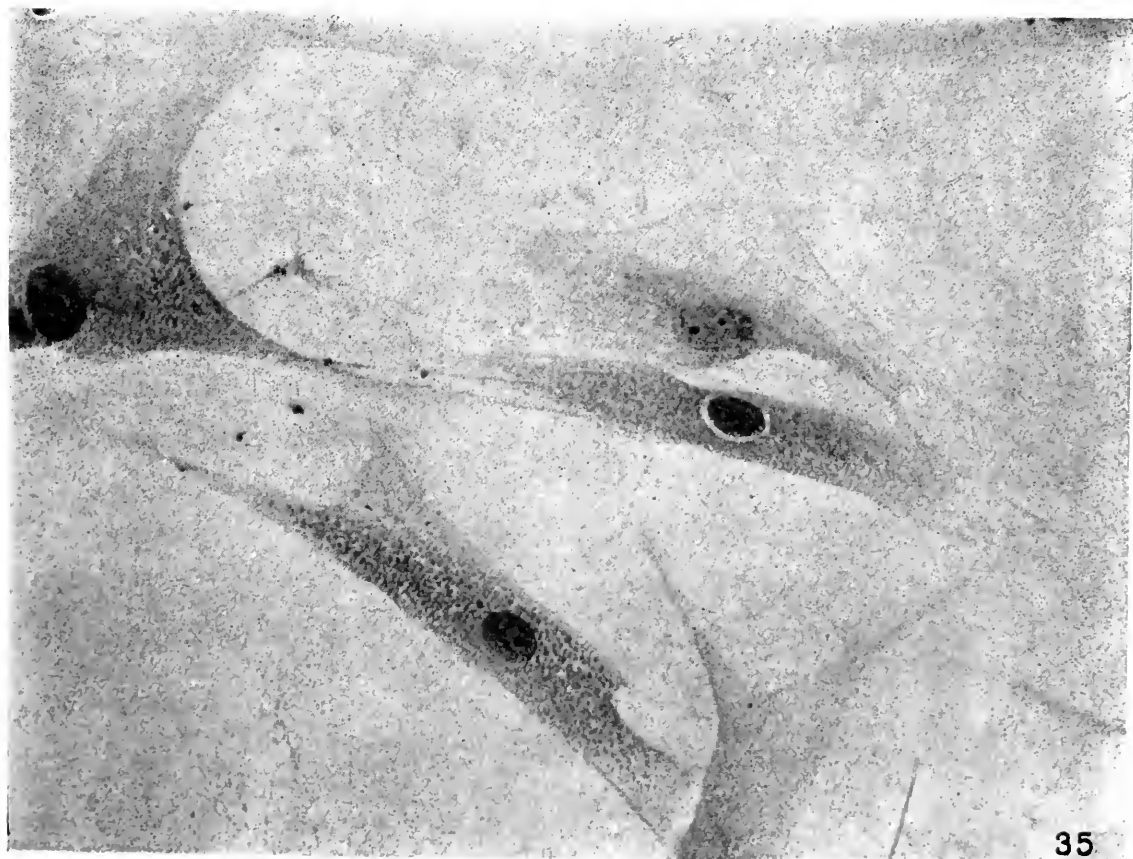
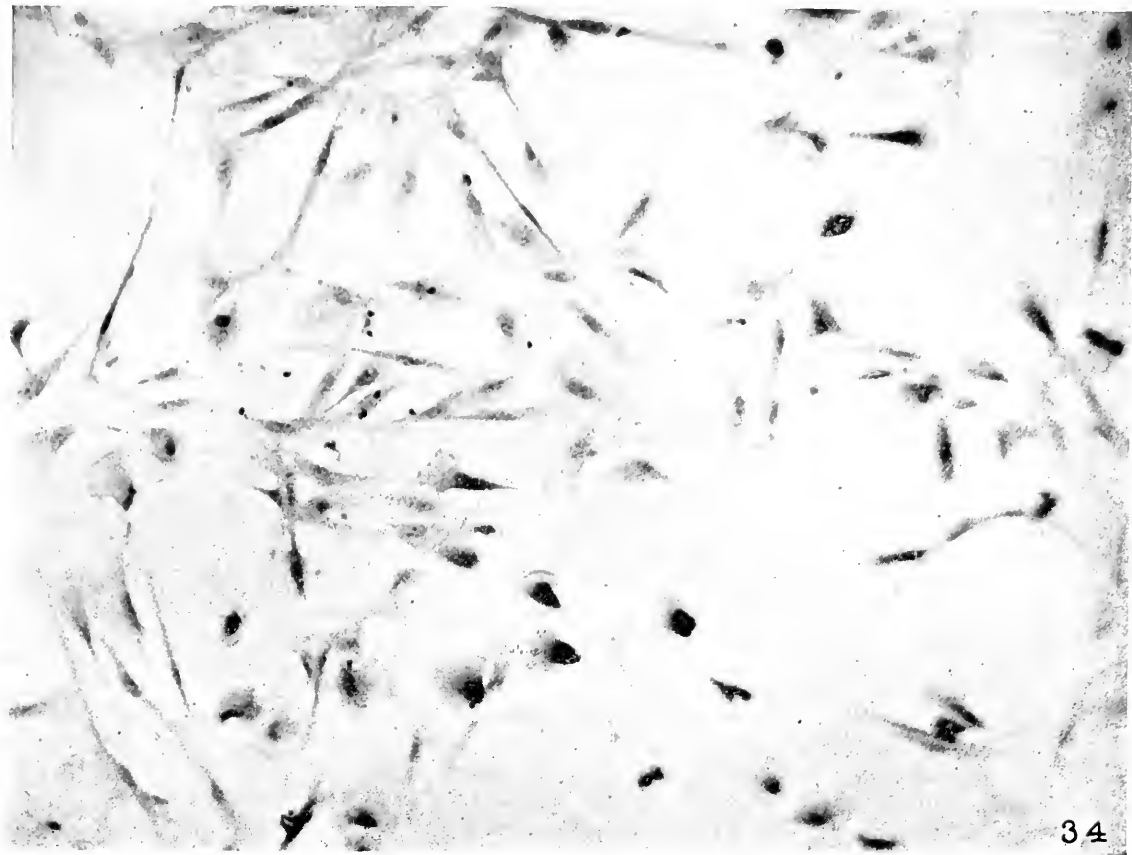
(Lewis and Webster: Cultures from human lymph nodes )





(Lewis and Webster: Cultures from human lymph nodes.)





(Lewis and Webster: Cultures from human lymph nodes,)





# THE INFLUENCE OF THYROID PRODUCTS ON THE PRODUCTION OF MYOCARDIAL NECROSIS.

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PLATE 33.

(Received for publication, May 11, 1921.)

In a recent study of hearts from cases of hyperthyroidism dying of myocardial exhaustion, the writer found acute necrosis of cardiac muscle, in one instance so diffuse as to involve a large part of the left ventricular wall.<sup>1</sup> The character of necrosis was that usually associated with extreme intoxication by acute infections such as diphtheria or scarlet fever, and more commonly occurring in youth. Since there was no evidence of such a severe intoxication in these cases it seemed probable that the myocardium was in a condition which rendered it much more easily injured than usual.

Inasmuch as practically every case of hyperthyroidism at one time or another shows cardiac symptoms, which not infrequently progress in severity until the patient becomes permanently incapacitated by myocardial exhaustion, it is generally considered that products of the diseased thyroid gland are the immediate cause of injury to the heart, though the manner in which their action is exerted is not understood.

The presence of destructive lesions of the myocardium in cases of exophthalmic goiter has been demonstrated by Fahr,<sup>2</sup> who attributes the injury to direct action of products of the diseased thyroid gland upon heart muscle fibers.

In the cases of hyperthyroidism studied by the writer there was no indication of an infection of sufficient virulence to be alone responsible for the necrosis. It was difficult also to think that so acute and uniform a lesion was the outcome of a slowly progressive degeneration

<sup>1</sup> Goodpasture, E. W., *J. Am. Med. Assn.*, 1921, lxxvi, 1545.

<sup>2</sup> Fahr, Th., *Centr. allg. Path. u. path. Anat.*, 1916, xxvii, 1.

of the myocardium resulting from continued action of products of a diseased thyroid, although such an explanation might suffice to account for the general disease of the heart which had persisted over a considerable period of time.

It seemed reasonable to suppose that these hearts overstimulated by disease of the thyroid and laboring in a condition bordering on exhaustion, were in a state of greater susceptibility to injury by toxic substances such as may have resulted from a relatively mild terminal infection which under other circumstances would not have materially injured the myocardium.

The following study was undertaken to determine, first, what demonstrable effect feeding desiccated thyroid gland, or intravenous administration of crystalline thyroxin would produce in the myocardium; then, whether the effect of these substances would cause the heart to be more readily injured by toxic agents, notably chloroform.

#### *Material.*

Rabbits free of infection were used for the experiments. It was found most satisfactory to use young adult males weighing from 2,000 to 2,500 gm. Unless animals of approximately the same age, weight, and condition were used there was a great deal of variation in the reaction of different individuals to the same dosage of thyroid products. These substances are more toxic for young rabbits than for old ones, and for rabbits of the same age and weight they seem less toxic during pregnancy, though the fetuses are readily killed.

In the first set of experiments two samples of desiccated thyroid gland containing 0.2 per cent iodine were used. The desired amount was mixed with water, and the thin paste formed was administered orally by means of a medicine dropper inserted far back in the mouth.

Crystalline thyroxin (Squibb's) containing about 65 per cent iodine was likewise used but was given by intravenous injection. 10 mg. were dissolved in 5 cc. of distilled water to which 2 drops of 10 per cent sodium hydroxide had been added. The solution was then sterilized in boiling water and used up within 2 or 3 days. In preparations kept longer, not infrequently a granular precipitate appeared, but until this happened there did not seem to be any diminution in the effectiveness of the solution.

In the second set of experiments rabbits treated with dried thyroid or crystalline thyroxin received chloroform by inhalation in addition. Chloroform (U.S.P.) for anesthesia was used for this purpose.

### *Method.*

Rabbits were kept in the laboratory several days on a diet of oats, hay, and carrots or spinach until their weight became constant or began to increase. Daily weight and pulse rate were recorded, and symptoms noted throughout the experiment. The first set, *i.e.* those receiving dessicated thyroid, received 1 gm. daily, suspended in water, administered through a medicine dropper. Those receiving thyroxin were given every 2nd or 3rd day 1 mg. dissolved in 0.5 cc. of alkaline solution and injected into an ear vein.

The pulse was counted by means of a stethoscope applied to the chest while the animal was quiet, and in some instances electrocardiographic tracings were made. As the rate sometimes rose to 400 or more per minute, it was often necessary to employ the method of dotting paper with a pencil, thus recording each impulse for 30 seconds to 1 minute and counting them afterward, a procedure shown by Levine to be very accurate.<sup>3</sup> The animals soon become accustomed to being handled and very uniform results were obtained. The animals in the first set, which received desiccated thyroid gland or thyroxin alone, were treated for from 2 to 3 weeks, and except in an occasional instance they were killed by a blow on the head and the tissues were fixed immediately. The average normal pulse rate for rabbits is around 200 per minute, though individuals show considerable variation. In the second group of experiments when the pulse rate rose to about 300 per minute the rabbits were given chloroform by inhalation for 1 hour and were killed by a blow on the head 24 to 72 hours later. Chloroform was given by the drop method or by holding the open mouth of the bottle above the nose just far enough removed to prevent spasmodic inhibition of respiration. The animals were never deeply anesthetized, the corneal reflex was not lost, and after the anesthetic was removed they immediately got on their feet.

<sup>3</sup> Levine, S. A., *J. Am. Med. Assn.*, 1920, lxxv, 795.

Tissues were fixed in Zenker's solution and stained by the eosin-methylene blue method for routine microscopic examination, and in other fixatives for special studies.

*The Effect of Desiccated Thyroid and Thyroxin.*

While it is appreciated that neither desiccated thyroid nor thyroxin may contain or represent the real secretion product of the thyroid gland in health or disease,<sup>4</sup> they both induce physiological activities more or less analogous to those present in patients with hyperthyroidism; namely, tachycardia, increased metabolism, nervousness, etc. It is estimated regarding dosage that 1 gm. of desiccated thyroid gland containing 0.2 per cent iodine under favorable conditions of preparation will contain approximately the equivalent of 1 mg. of thyroxin. I have found, however, with the preparations used in rabbits that 1 mg. of thyroxin injected intravenously is considerably more potent in increasing the pulse rate and inducing loss of weight than 1 gm. of desiccated thyroid administered by mouth.

There is a great deal of variation in the way different rabbits react to the same dosage. Old individuals are very resistant to the quantities which were employed; younger ones vary in susceptibility according to age, weight, and general condition. The more refractory animals gradually acquire a tolerance to large doses, while the susceptible ones rapidly emaciate and die, apparently from thyroxin intoxication. They show toward the end extreme muscular weakness, being unable to hold up their heads. Diarrhea is a frequent early symptom and may persist, but often disappears on continued treatment. As was noted by Wilson and Kendall,<sup>5</sup> an animal will sometimes recover from a serious degree of intoxication even though it continues to receive large doses of thyroxin. The rabbits eat very little for the first few days and those most intoxicated abstain from food almost entirely, which explains largely the rapid loss in weight. As treatment continues, frequently the hair becomes dry,

<sup>4</sup> The delayed action of these substances does not harmonize with the rapid effects produced by Cannon on stimulation of the thyroid, and attributable to functional activity of this gland.

<sup>5</sup> Kendall, E. C., *Tr. Assn. Am. Phys.*, 1915, xxx, 420. Wilson, L. B., and Kendall, E. C., *Tr. Assn. Am. Phys.*, 1915, xxx, 458.

lusterless, and tends to fall out. Excitability and restlessness increase. No sugar has been found in the urine.

The two effects which I have followed most carefully are those on pulse rate and body weight. The more constant of the two is an increased heart rate which practically always occurs to a greater or less degree with or without loss in weight. There is no constant relation between the two. The cardiac rhythm has always remained regular. Under the influence of excitement or exercise the rate rapidly increases, sometimes 25 per cent, but subsides within a few minutes to a constant level.

The rise in pulse rate begins 24 to 48, sometimes 72 hours after the initial dose, somewhat sooner after thyroxin than after desiccated thyroid. It rapidly increases to 50 or 75 per cent above the normal rate, and maintains a high level under treatment. The pulse becomes more forceful and the ear veins appear to dilate and contract more readily than normally. No constant elevation of temperature has been noted.

A progressive secondary anemia accompanies the severer degrees of intoxication.

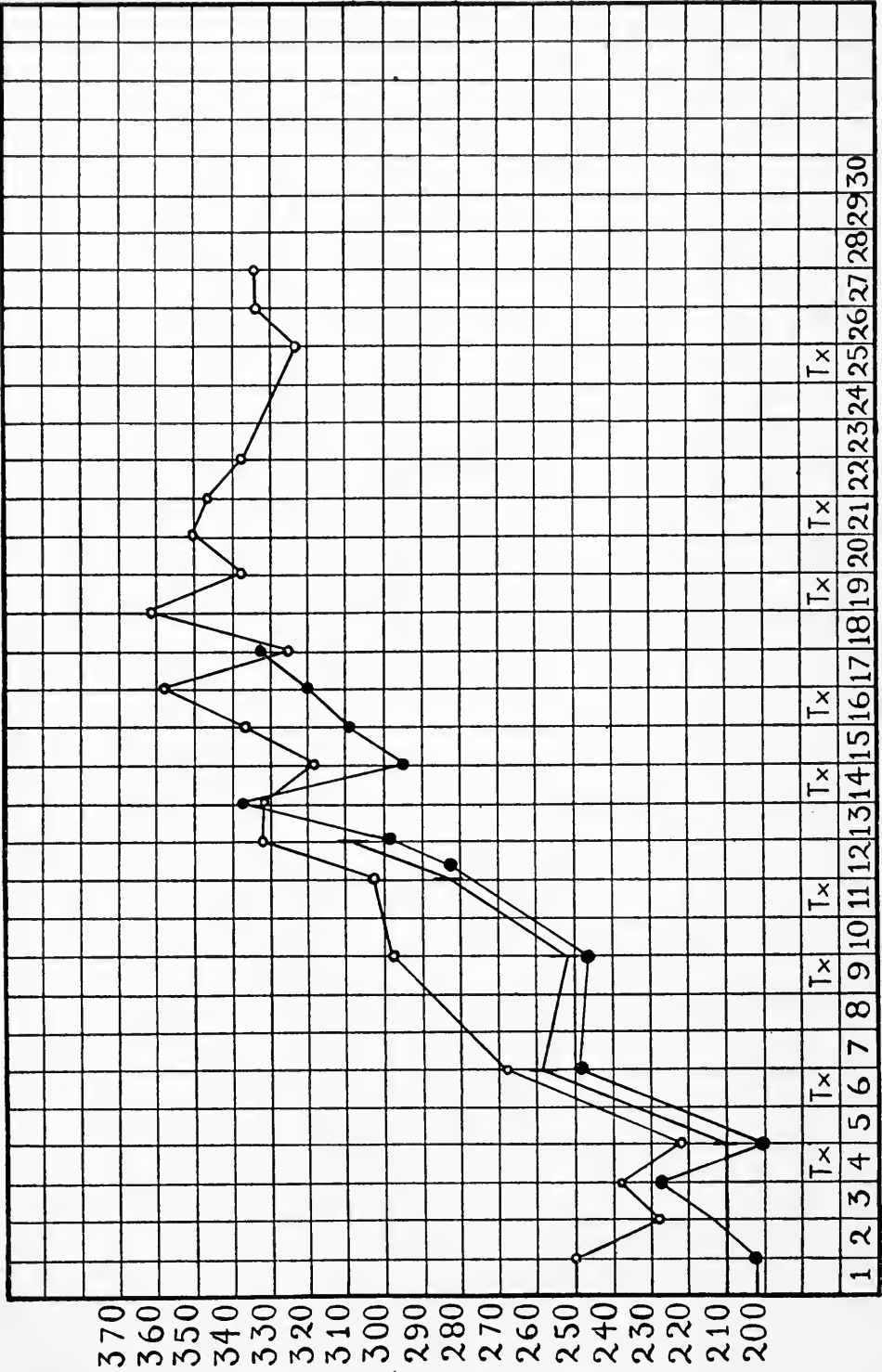
Text-figs. 1 and 2 illustrate the relative increase in pulse rate and loss of weight in the same group of animals after thyroxin administration.

To observe the changes produced in the myocardium by feeding thyroid gland the hearts of seven rabbits were studied. Two had received 1 gm. of thyroid daily for 15, two 16, one 17, one 19, and one 26 days. A typical protocol follows:

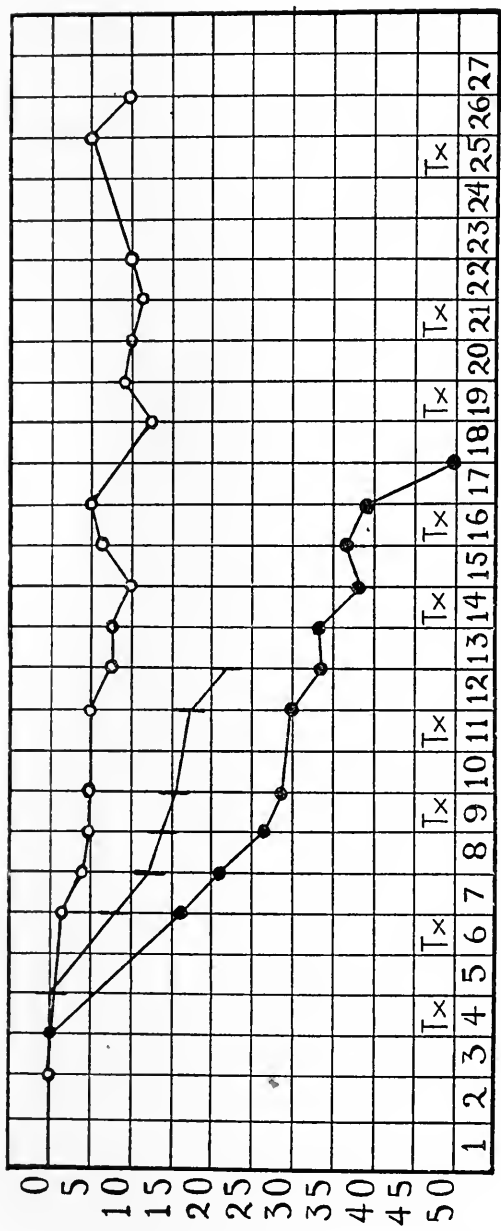
*Rabbit 1.*—Brown; young adult male.

Aug. 19, 1920. Weight 2,322 gm.; pulse 170.

" 20.	" 2,361	" "	" 154.	
" 21.	" 2,380	" "	" 156.	
" 23.	" 2,446	"		thyroid 1 gm.
" 24.	" 2,375	" pulse 176;	" 1	"
" 25.	" 2,267	" " 188;	" 1	"
" 26.	" 2,206	" " 204;	" 1	"
" 27.	" 2,132	" " 206;	" 1	"
" 28.		" 256;	" 1	"
" 30.	Weight 1,997 gm.;	" 244;	" 1	"
" 31.	" 1,970	" 272;	" 1	"



TEXT-FIG. 1. Increase in pulse rate following the intravenous injection of crystalline thyroxin. The abscissæ indicate days, the ordinates pulse rate.  $T_x$ , received 1 mg. of thyroxin crystals. |—|—| Average increase in five rabbits. ○—○—○ Increase in Experiment 2. ●—●—● Increase in Experiment 4.



TEXT-FIG. 2. Percentage loss in weight following the intravenous injection of crystalline thyroxin.  $T_x$ , received 1 mg. of thyroxin. The abscissæ indicate days, the ordinates percentage loss in body weight. —|—| Average per cent loss in body weight. O—O—O Per cent loss in Experiment 2. ●—●—● Per cent loss in Experiment 4. The same group as in Text-fig. 1.

Sept. 1, 1920.	Weight	1,933 gm.;	pulse	250;	thyroid	1 gm.
" 2.	"	1,922 "	"	262;	"	1 "
" 3.	"	1,885 "	"	308;	"	1 "
" 4.	"	1,868 "	"		"	1 "
" 7.	"	1,911 "	pulse	268;	"	1 "
" 8.	"	1,905 "	"		"	1 "
" 9.	"	1,878 "	"		"	1 "
" 10.	"	1,877 "	"		"	1 "
" 11.	"	1,852 "	"		"	1 "
" 13.	"	1,928 "	"		"	1 "
" 15.			pulse	312;	"	1 "
" 17. Killed. Tissues fixed in Zenker's solution. Lungs normal. Heart shows no gross abnormality. Other organs show no gross changes.						

The hearts from a similar group of rabbits that had received thyroxin intravenously over periods of 9, 14, 18, 20, and 23 days respectively were studied in the same way. The following protocol is an instance of extreme intoxication, the animal being killed in an almost moribund condition.

*Rabbit 2.*—White; young adult male.

Oct. 7, 1920.	Weight	2,045 gm.;	pulse	200.
" 8.	"	2,180 "	"	226.
" 9.	"	2,141 "	"	232.
" 11.	"	2,222 "	"	200; thyroxin 1 mg.
" 13.	"	2,010 "	"	240; " 1 "
" 14.	"	1,980 "		
" 15.	"	1,940 "		
" 16.	"	1,985 "	pulse	212; thyroxin 1 mg.
" 18.	"	1,867 "	"	244; " 1 "
" 19.	"	1,750 "	"	276; " 1 "
" 20.	"	1,665 "	"	312; " 1 "
" 21.	"	1,603 "	"	292; " 1 "
" 22.	"	1,595 "	"	272; " 1 "
" 23.	"	1,525 "	"	300; " 1 "
" 24.			"	272; " 1 "
" 25.	Weight	1,405 gm.;	"	268; " 1 "
" 26.	"	1,468 "	"	220; " 1 "
" 27.	"	1,422 "	"	252; " 1 "
" 28.	"	1,330 "	"	240; " 1 "
" 29.	"	1,221 "	"	256; " 1 "
" 30. Very weak, especially muscles of neck. Head bends over and animal cannot raise it. Greatly emaciated. Killed.				



*Autopsy.*—Lungs clear. Heart weight 4.75 gm. No gross changes. Other organs small and atrophic. Stomach almost empty. Brownish blood streaks and small erosions of mucosa with edema of wall in places. Adrenals smaller than normal, pink and meaty, with no obvious cortical fat. Spleen small and brownish. Tissues fixed in Zenker's solution.

Four of the five rabbits which received similar doses of thyroxin were intoxicated in the above manner. One was relatively resistant and although it showed a marked increase in pulse rate, there was little loss in weight.

None of the hearts from these two groups of animals, *i.e.* the one receiving desiccated thyroid, the other crystalline thyroxin, showed any gross lesion excepting atrophy in the case of those animals which rapidly emaciated while receiving thyroxin. Numerous sections were cut from both ventricles and auricles. Microscopically definite lesions of a relatively insignificant nature were found. They consisted in a perivascular fibrosis with some muscle destruction about small arteries and, less frequently, veins, situated especially in the wall of the right ventricle (Fig. 1). More rarely there was a small area of fibrosis at the base of a papillary muscle or within the wall of the left ventricle. The auricles showed no abnormality. Most of these lesions were healed and must have occurred soon after the first doses were given. They appeared somewhat more pronounced in the group which received desiccated thyroid. In the heart of one animal that died of thyroxin intoxication 9 days after the initial dose there were very acute small focal necroses about the wall of veins in the right ventricle. These lesions evidently occurred a few hours before death.

Altogether the effect of stimulation of the heart by thyroid products appeared to be injurious to the myocardium following the first doses, but no lesions indicated that this effect was progressive. The very definite relation of muscle injury and repair to blood vessels, both arteries and veins, suggests the possibility of a mechanical basis of origin, depending upon alterations in the circulation of blood rather than a direct toxic action of the thyroid products upon the muscle cells. This view is strengthened by the characteristics of the lesion in certain instances in which there does not appear to be a primary necrosis of muscle cells, but an interstitial fibrosis about the vessels.

*The Effect of Chloroform on the Myocardium Stimulated by Desiccated Thyroid or Thyroxin.*

The above experiments formed the basis and control for the following study to determine the susceptibility of the myocardium of animals stimulated by thyroid products to injury by known toxic substances, notably chloroform. While the toxic action of chloroform on the heart is well known, light anesthesia for an hour on 2 or 3 successive days in normal rabbits does not produce necrosis of the myocardial cells, as was shown in ten control animals. It has been found, however, that a similar procedure with rabbits that have received desiccated thyroid or thyroxin for several days until their heart rate becomes accelerated to about 300 beats per minute will usually produce necrosis of the myocardium, especially of the left ventricular wall, and often the destruction is very severe and diffuse. It is necessary in order to obtain more uniform results that chloroform be administered by inhalation, for while the total quantity of the drug absorbed by this method is much less than was introduced for comparison by subcutaneous injections in olive oil or by stomach, the effects on the heart muscle are very much greater. This is to be explained, I think, by a natural assumption that a greater concentration of chloroform during the period of inhalation acts upon the heart than is the case at any time when the drug is absorbed through the other routes. Following chloroform inhalation in these animals there is usually no central necrosis of the liver, even when a very extensive necrosis of myocardium results, whereas after the administration of 0.2 cc. of chloroform per kilo in equal quantity of olive oil subcutaneously the animal will die usually within 24 hours, with extreme central necrosis of the liver and little or no necrosis of heart muscle. Ordinarily the liver is more readily injured by chloroform inhalation than is the cardiac muscle and the only explanation of the absence of central hepatic necrosis in the experiments on hyperthyroidism animals is that the cardiac muscle is rendered, by the administration of thyroid products, more easily injured than the normal heart, and that an insufficient quantity of chloroform is inhaled to produce toxic necrosis of the liver. By the method of inhalation employed the animal is never deeply anesthetized. If

deep anesthesia is obtained the rabbits rarely live 12 hours and apparently die of cardiac failure. The amount of chloroform actually received by inhalation is regulated largely by reflex inhibition of respiration which is quite easily induced in these animals and is attended by inhibition of the heart. If the animals are not sufficiently anesthetized to lose this reflex, they are safe from immediate untoward effects.

In the experiments to test the effect of chloroform on the myocardium, young rabbits weighing from 1,800 to 2,500 gm. were used. They received a diet of oats, hay, carrots, and spinach. Desiccated thyroid gland was given to three animals in 1 gm. daily doses for periods of 12, 16, and 17 days respectively, then chloroform by inhalation for 1 hour. They were killed 24 to 48 hours later by a blow on the head, and tissues fixed immediately. A sample protocol follows:

*Rabbit 3.*—Brown; young adult male.

Aug. 19, 1920. Weight 3,325 gm.; pulse 208.

“ 20. “ 3,281 “ “ 206.

“ 21. “ 3,157 “ “ 180.

“ 23. “ 3,245 “ thyroid 1 gm.

“ 24. “ 3,248 “ pulse 224; “ 1 “

“ 25. “ 3,195 “ “ 206; “ 1 “

“ 26. “ 3,145 “ “ 224; “ 1 “

“ 27. “ 3,041 “ “ 254; “ 1 “

“ 28. “ “ “ 262; “ 1 “

“ 30. Weight 2,785 gm.; “ 224; “ 1 “

“ 31. “ 2,800 “ “ 278; “ 1 “

Sept. 1. “ 2,758 “ “ 314; “ 1 “

“ 2. “ 2,792 “ “ 300; “ 1 “

10.45 a.m. Light chloroform anesthesia for 1 hour. Pulse rate during anesthesia: 11 a.m. 374; 11.25 a.m. 364; 11.45 a.m. 356. Got up immediately after chloroform was stopped.

Sept. 3, 9 a.m. Weight 2,675 gm.; pulse 316. Pulse weak and absolutely irregular. Thyroid 1 gm. 4 p.m. Pulse 448; irregular.

Sept. 4, 11 a.m. Pulse 400. Very irregular and weak. Looks sick. Killed by blow on head.

*Autopsy.*—Lungs clear, pink. Heart large, dilated; weight 10 gm. Walls thick and edematous. Both ventricular walls streaked and mottled with large opaque yellowish gray areas most marked in interventricular septum and right ventricle though present throughout both ventricles. Liver, central fatty change. Adrenals small.

*Microscopic Examination.*—Diffuse acute necrosis of myocardium of both ventricles.

This animal showed a much more extensive necrosis than the other two in which microscopic acute focal necroses of muscle cells were present in each ventricle. So extensive a necrosis has not been duplicated in rabbits receiving thyroxin.

Because of the complexity and lack of uniformity of desiccated thyroid gland, crystalline thyroxin was used in the subsequent experiments, twelve in number. Doses of 0.5 to 1 mg. were injected into an ear vein, usually every 2nd day, chloroform inhalation for an hour was given later, from 2 to 23 days after the initial injection. In some instances chloroform inhalation was repeated on the 2nd or 3rd day after the first inhalation. The animals were killed 24 to 72 hours after treatment with chloroform. The results of these experiments are given in Table I.

*Rabbit 4.*—Young, gray, three-fourths grown male.

Mar. 1, 1921. Weight 1,840 gm.; pulse 230; thyroxin 1 mg.

" 2. " 1,695 " " 290; diarrhea.

" 3. " 1,610 " " 320; "

9.50 a.m. Light chloroform inhalation, 1 hour. Pulse during inhalation: 10 a.m. 296; 10.10 a.m. 296; 10.20 a.m. 288; 10.30 a.m. 304; 10.40 a.m. 280; 10.50 a.m. 275. Got up immediately after chloroform was removed.

Mar. 4. Weight 1,550 gm.; pulse 348. No evident diarrhea. Pulse regular, strong. Thyroxin 0.5 mg.

Mar. 5. Weight 1,480 gm.; pulse 360. Stools soft.

Mar. 6. Weight 1,450 gm.; pulse 348; thyroxin 0.5 mg.

Mar. 7. Weight 1,370 gm.; pulse 364. Light chloroform inhalation for 1 hour. Pulse during inhalation: 10 a.m. 376; 10.10 a.m. 360; 10.20 a.m. 384; 10.30 a.m. 340; 10.40 a.m. 368; 10.50 a.m. 356; 11 a.m. 380. Very lightly under; came out immediately.

Mar. 8. Weight 1,230 gm.; pulse 340. Heart beat weak but regular, even after struggling for 2 minutes. There seem to be two rhythms, palpable over heart, one more rapid at base. Electrocardiogram shows regular beat. 3.30 p.m. Killed by blow on head.

*Autopsy.*—Ventricles collapsed and fibrillating. Large opaque yellowish patches in wall of left ventricle. Small opaque dots in right. Lungs, emphysematous; fresh focal hemorrhages posteriorly in both lower lobes. (Both emphysema and fresh peribronchial hemorrhages occur in these animals as a result of sudden blow on head. In one instance rupture of the liver was found.)

*Microscopic Examination.*—Diffuse necrosis of left and focal necrosis of right ventricle were found. No necrosis of liver.

TABLE I.

*Intravenous Injections of Crystalline Thyroxin Followed by Chloroform Inhalation.*

Animal No.	Weight.	Amount of thyroxin.	Duration of treatment.	Period of chloroform inhalation.	Total length of experiment.	Results.
	<i>gm.</i>	<i>mg.</i>	<i>days</i>		<i>days</i>	
5	2,652	4.5	6	1 hr.	10	Killed 4 days after chloroform. Diffuse necrosis in left ventricle, focal in right.
6	2,195	7	6	1 “	7	Killed 3 days after chloroform. Diffuse necrosis in left ventricle.
7	2,493	6	11	1 “ each 2 days.	12	Killed 1 day after last chloroform inhalation. No necrosis.
8	1,695	3	4	1 “	4	Died 6 hrs. after chloroform. No necrosis.
9	1,432	3	4	1 “	4	Died 8 hrs. after chloroform. No necrosis.
10	2,447	3	4	1 “	5	Killed 1 day after chloroform. Focal necrosis.
11	2,500	6	10	1 “	11	Died 18 hrs. after chloroform. Focal necrosis.
12	2,400	4	7	1 “ each 2 days.	10	Killed 3 days after first chloroform inhalation, 1 day after last. Diffuse necrosis.
4	1,840	2	2	1 “ “ 2 “	6	Killed 1 day after last chloroform inhalation, 5 days after first. Diffuse necrosis.
13	1,815	3	8	1 “	9	Killed 1 day after chloroform. Focal necrosis.
14	2,190	4	8	1 “	9	Killed 1 day after chloroform. Focal necrosis.
15	1,800	3	9	1 “	10	Killed 1 day after chloroform. Diffuse necrosis.

In none of these animals was there evidence of sufficient chloroform poisoning to have caused death had they not been killed, and undoubtedly all would have recovered, although in some instances

as much as one-fourth of the left ventricular myocardium appeared to have been destroyed. The lesions, however, were uniform, not progressive, and healing ensued with surprising rapidity, consequently if the rabbit survived the first 24 hours after chloroform the worst effects were safely over.

In five of the twelve rabbits that received thyroxin and chloroform there were large macroscopic areas of necrosis in the left ventricle and focal microscopic areas in the right; in three were microscopic foci in both left and right ventricles, and in three no necrosis was found, two of these having died within 24 hours after chloroform. No lesions were found in the auricles in any of these animals.

#### *Characteristics of the Lesion.*

In order to avoid the possibility of confusing any lesions produced experimentally with a preexisting scarring of the myocardium (a condition, however, not seen in any of the controls), the rabbits have been killed at short intervals after the inhalation of chloroform. In this way it has been possible to study the acute changes and to follow the process of repair. It is of interest that in no instance has simple fatty degeneration without necrosis been found in the animals which received thyroid products either alone or with chloroform. Fatty degeneration of the myocardium is the commonest pathological change noted in hearts from cases of exophthalmic goiter, yet it was not present in the two cases of hyperthyroidism reported by the writer except as an incident in necrosis. So in the experimental lesions cells in the neighborhood of necrotic areas contain an excess of fat, but it is quite evident that fatty degeneration did not precede the necrosis. In one instance there were patches of fatty degeneration with relatively little necrosis corresponding in distribution to necrotic areas in other hearts. This change obviously indicated injury but not sufficient to result in cellular death. The necrotic cells contain fractured bundles of fibrillæ, granules of various kinds, and often irregular transverse bands of hyaline material. The dead cells within a few hours become surrounded and invaded by large mononuclear phagocytes which are found in very great numbers within 48 hours (Fig. 2), incorporating particles of the disintegrating cells. At this time the fibroblasts are also extremely active, proliferat-

ing *in situ* and wandering in from the adventitia of neighboring blood vessels. Mitotic figures are present in great numbers. Polymorphonuclear leucocytes play no part in the lesion.

At the end of 72 hours (Figs. 3 and 4) small lesions have been replaced by young fibroblasts and in another 24 hours the lesions are practically completely healed by a loose scar tissue in which no wandering cells remain. Considerable edema accompanies the lesions but no hemorrhages or vascular changes have been observed. There is to be detected often a very definite perivascular distribution of necrosis, especially in the right ventricle. It may be found in connection with both arteries and veins. No evidence whatever of regeneration of cardiac muscle cells has been observed. In one instance fusion of mononuclear phagocytes occurred, forming giant cells similar in appearance to those of the Aschoff bodies appearing in myocarditis of rheumatic fever.

#### DISCUSSION.

It has been demonstrated by postmortem studies upon the human heart that a severe terminal acute necrosis of the cardiac muscle may occur in cases of hyperthyroidism dying of myocardial exhaustion without the usual evidences of profound intoxication with which this lesion is commonly associated. It seemed likely that these hearts, overstimulated by disease of the thyroid gland and laboring in a condition bordering on exhaustion, were in a state of greater susceptibility to injury by toxic substances such as may have resulted from a relatively mild terminal infection, or other sources, which under ordinary circumstances would not have materially injured the myocardium.

In the foregoing experiments a study has been made of the demonstrable effects of large doses of dried thyroid gland and of thyroxin upon the myocardium of rabbits. In brief it has been shown that rabbits receiving daily 1 gm. of thyroid gland or 1 mg. of crystalline thyroxin intravenously every 2nd day exhibit a marked increase in pulse rate, more forceful action of the heart, loss in body weight, increasing irritability, and frequently diarrhea and falling out of hair. Animals subjected to this treatment alone and killed at periods of 2 to 3 weeks may show relatively slight though definite lesions in

the myocardium, notably perivascular necrosis or fibrosis in the wall of the right ventricle, focal necrosis or fibrosis in papillary muscles of the left ventricle, and, more rarely, scattered small focal necroses within the myocardium elsewhere. These lesions evidently occur early in the treatment, for at the time examinations were made they were for the most part healed, and no evidence of progression under continued administration of thyroid products was observed.

The second series of experiments to test the susceptibility of the myocardium of similarly treated animals to injury by chloroform has yielded more striking results; that is to say, rabbits subjected to the feeding of 1 gm. of desiccated thyroid daily or receiving 1 mg. of crystalline thyroxin every 2nd day until their pulse rate reaches about 300 per minute, show in the majority of instances, following one or two light anesthetics from inhalation of chloroform vapor for 1 hour, a widespread necrosis of myocardium, sufficient in one instance to cause severe disturbances of cardiac function.

These results seem to support the view that a heart stimulated to an abnormal activity by products of the thyroid gland may be more readily injured than the normal heart; and they suggest a possible explanation for the severer degrees of cardiac disease in association with hyperthyroidism. One may well imagine that in the early stages of hyperthyroidism, when the heart is overstimulated by products of the diseased thyroid, acute infections or intoxications which would leave no significant impress on the normal person may be permanently injurious to the heart thus affected and with repetition of the injury the cardiac condition may become progressively worse.

The evidence does not indicate that products of the thyroid alone could be entirely responsible for the cardiac lesions which may occasionally occur in man in association with hyperthyroidism.

The immediate cause for the increased susceptibility to injury which these hearts exhibit is speculative. It occurs to us that in the state of hyperthyroidism and in the experimental condition following the administering of thyroid products, the nutritional reserve of the myocardium may be diminished, especially glycogen. Hearts of experimental animals in which cardiac necrosis has been produced show no glycogen microscopically, but whether a diminution in the amount of stored glycogen would predispose to injury is uncertain.



## SUMMARY.

An experimental study upon the demonstrable effects of large doses of dried thyroid gland and thyroxin upon the myocardium of rabbits has been made. Animals under such treatment show characteristic clinical symptoms with definite, although relatively slight, myocardial lesions. Similarly treated animals which have, in addition, been subjected to chloroform anesthesia show more striking, widespread myocardial necrosis.

These experiments indicate very distinctly that chloroform as an anesthetic in cases of hyperthyroidism is apt to be exceptionally detrimental to the myocardium, and should be avoided.

## EXPLANATION OF PLATE 33.

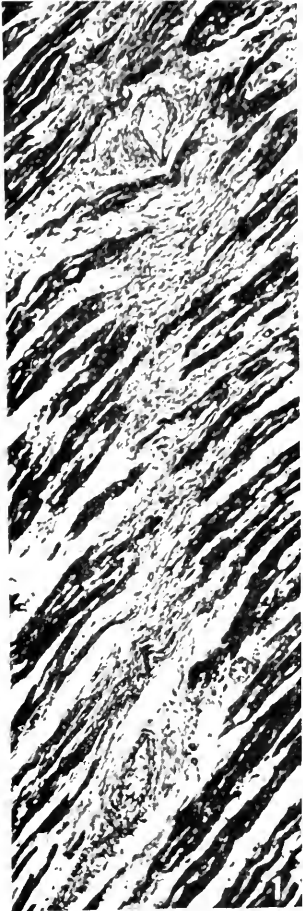
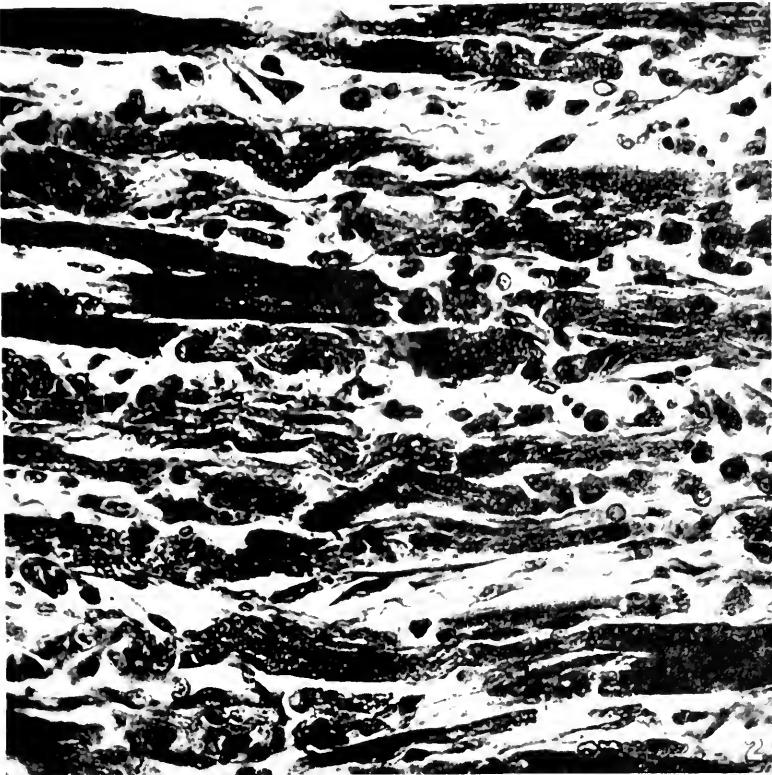
FIG. 1. Perivascular fibrosis in right ventricle following intravenous injection of thyroxin.

FIG. 2. Acute necrosis; 48 hours. Desiccated thyroid plus chloroform.

FIG. 3. Acute necrosis; 72 hours. Showing fibroblastic proliferation. Thyroxin plus chloroform.

FIG. 4. Acute necrosis; 72 hours. Thyroxin plus chloroform.

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(Goodpasture: Myocardial necrosis.)



## CICATRIZATION OF WOUNDS.

### XII. FACTORS INITIATING REGENERATION.

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(Received for publication, May 24, 1921.)

The nature of the factors which initiate cicatrization after an injury is not as yet exactly known. The resumption of cell proliferation in the wounded tissues of an adult animal may be attributed to the removal of resistance to growth, in consequence of the defect resulting from loss of tissue.<sup>1</sup> In other words, the removal of the products of growth, that is of a portion of the tissues, immediately reinaugurates the growth process, just as the removal of the products of a balanced chemical reaction at equilibrium immediately re-initiates the forward reaction.<sup>2</sup> This means that regeneration, being a direct consequence of the injury, is started by forces within the organism. But the same phenomenon may also be logically attributed to the action of an external factor. According to this hypothesis, the cells would be directly stimulated to growth and multiplication by forces without the organism, acting on tissues deprived of their natural protection by the injury.

#### I.

##### *Effect of Protection against Irritation.*

If regeneration is a direct consequence of the loss of tissue and initiated by an internal factor, the cicatrization of a wound protected against all external irritation must take place normally. But if this hypothesis be not true, the wound should not begin to cica-

<sup>1</sup> Welch, W. H., *Science*, 1897, v, 813.

<sup>2</sup> Robertson, T. B., *Principles of biochemistry for students of medicine, agriculture and related sciences*, Philadelphia, 1920, 482.

trize. It was observed in 1908 that the latent period of cicatrization of a wound dressed with dead connective tissue or plasma clot was abnormally prolonged. This fact suggested that regeneration was not initiated directly by the loss of tissue and that, if the surface of the wound were effectively protected against mechanical, chemical, and bacterial irritations, the setting in motion of the process of cicatrization would be indefinitely postponed. In order to ascertain in what measure the onset of regeneration could be delayed by adequate protection of the surface of the wound, five experiments were performed. Two circular wounds of equal size were made on the dorsal region of dogs, according to a technique previously described.<sup>3,4</sup> The control wound was covered with a paste containing chloramine-T in a concentration which had been shown to be non-irritating for the tissues, and to keep them in a sterile condition.<sup>5</sup> The experimental wound was dressed with subcutaneous connective tissue, excised from the lumbar region of a dog and kept in cold storage. Circular flaps, slightly larger than the wound and about 0.5 cm. thick, were prepared and fixed to the surface of the experimental wound by a few stitches. Both wounds were protected by a pad of dry gauze, sutured to the skin. Then the dressing was completed by a few other gauze pads, a large amount of cotton, a bandage, and a shirt. The animals were examined after a period of time varying from 13 to 25 days. As the examination involved the removal of the stitches holding the inner dressing to the skin, and also of the stitches fixed to the connective tissue placed on the wound, necessitating a considerable disturbance of the wound, the experiment was stopped after the second or third dressing.

The results of the five experiments are summarized in Table I. Experiment 1 was unsuccessful because the gauze pads slipped from the surface of the wounds and infection occurred. 14 days after the operation, no difference was found in the condition of both control and experimental wounds. Experiments 2 and 3 succeeded partly. The protection given to the wounds by the connective

<sup>3</sup> Carrel, Alexis, and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429.

<sup>4</sup> All operations were performed under ether anesthesia.

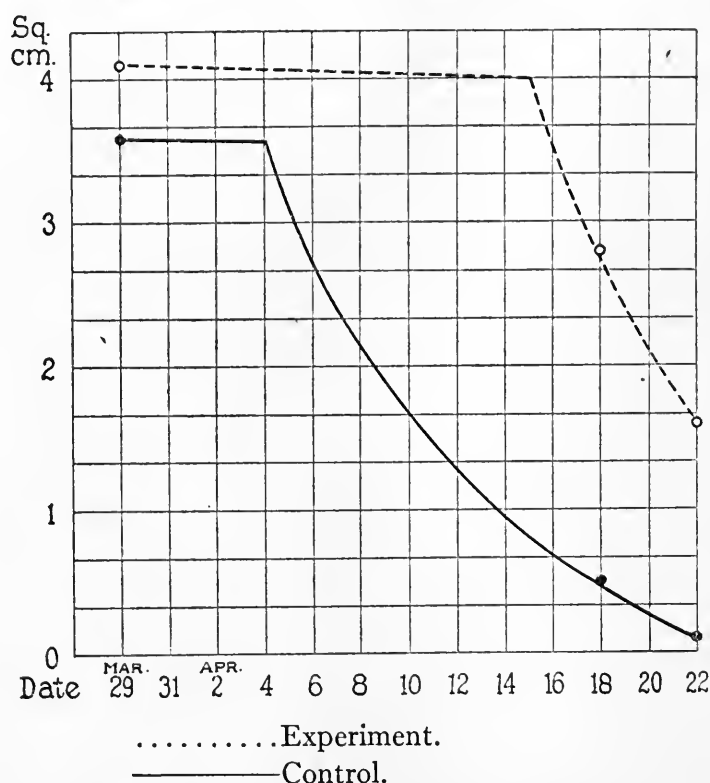
<sup>5</sup> Carrel, Alexis, du Noüy, P. L., and Carrel, Anne, *J. Exp. Med.*, 1917, xxvi, 279.

TABLE I.

*Action of Connective Tissue Dressing on the Latent Period.*

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
		1921	sq. cm.			sq. cm.			
1	1	Apr. 5	4.0		Chloramine-T.	4.6		Connective tissue.	Connective tissue was heated at 56°C. for 3 hrs.
		" 19	1.9	16	"	1.4	1		Inner dressing slipped from both wounds.
2	2	Mar. 29	3.6		"	4.1		Connective tissue.	Text-fig. 1.
		Apr. 18	0.5	0	"	2.8	0	Chloramine-T.	Inner dressing slipped from both wounds. Connective tissue dressing partially disappeared.
		" 22	0.1	0	"	1.6	0	"	
3	3	Mar. 31	4.0		"	3.9		Connective tissue.	Inner dressing slipped. Infection of both wounds.
		Apr. 19	0.7	∞		2.8	∞		
4	4	Mar. 31	3.5		Chloramine-T.	4.7		Connective tissue.	Text-fig. 2. No displacement of dressing.
		Apr. 7	3.0	0	"				
		" 19	2.0	0	"	4.7	0	Dry gauze.	
		" 25	1.7	0	"	5.1		" "	Appearance of granulation tissue.
5	5	" 7	5.2		"	4.2		Connective tissue.	Text-fig. 3. Connective tissue heated at 56°C.
		" 20	3.3	0	"	4.0	0	Chloramine-T.	No displacement of dressing.
		" 25	3.0	0	"	4.5	0		Appearance of granulation tissue.

tissue dressing was incomplete. When the wounds were inspected for the first time, 19 and 20 days respectively after the operation, the experimental wound was no longer covered by the connective tissue dressing, and cicatrization had started. In Experiment 2, the curve expressing the progress of regeneration showed that the latent period had lasted very much longer in the wound protected by connective tissue than in the control wound (Text-fig. 1). The duration of the latent period was probably 17 days, while in the control it was 6.

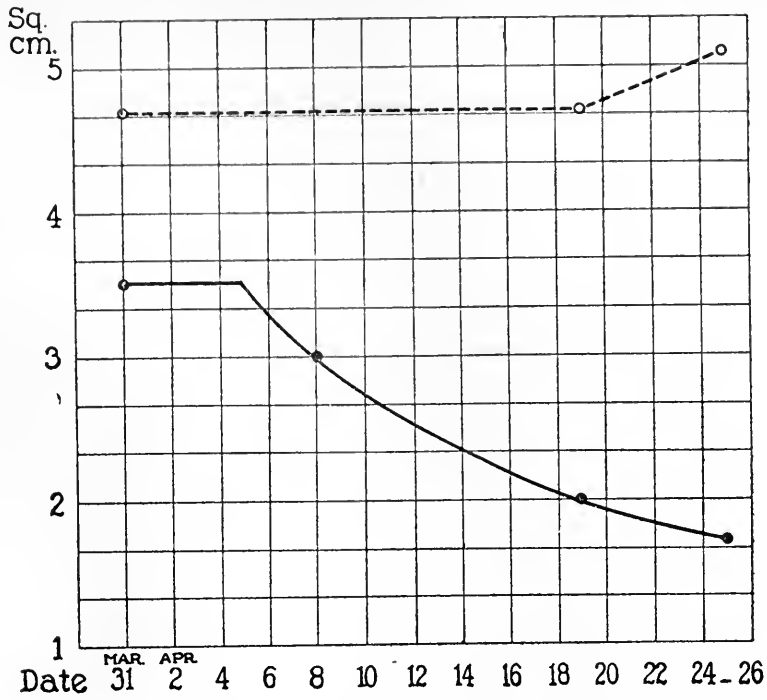


TEXT-FIG. 1. Experiment 2, Table I.

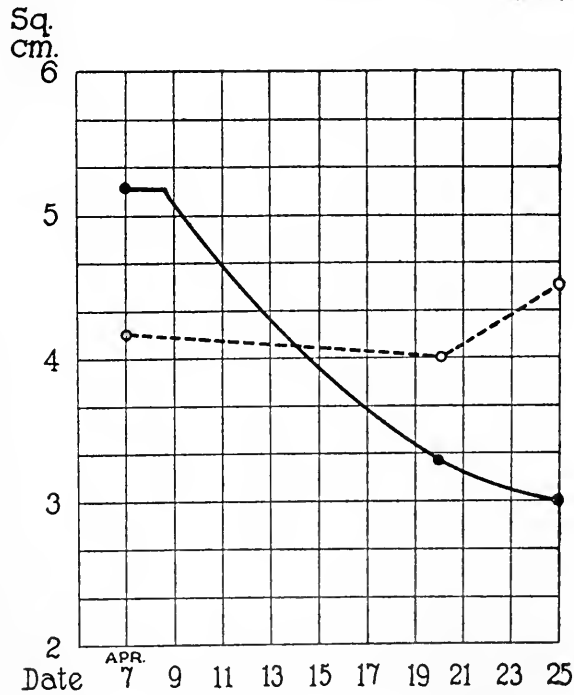
Although the connective tissue dressing did not remain at the surface of the wound for a long time, its effect was, however, manifest.

More significant results were obtained in Experiments 4 and 5. The connective tissue dressing remained exactly where it was applied and the surface of the wound was really protected against all outside irritation. The examination of the wounds was made 25 days after the operation in Experiment 4, and 18 days after the operation in Experiment 5. The period of contraction had not yet started and the area at that time was as large as at the time of the operation (Text-





TEXT-FIG. 2. Experiment 4, Table I.



TEXT-FIG. 3. Experiment 5, Table I.

figs. 2 and 3). It was a striking fact that a wound, effectively protected by a non-irritant dressing, did not show any evidence of cicatrization 25 days after the operation.

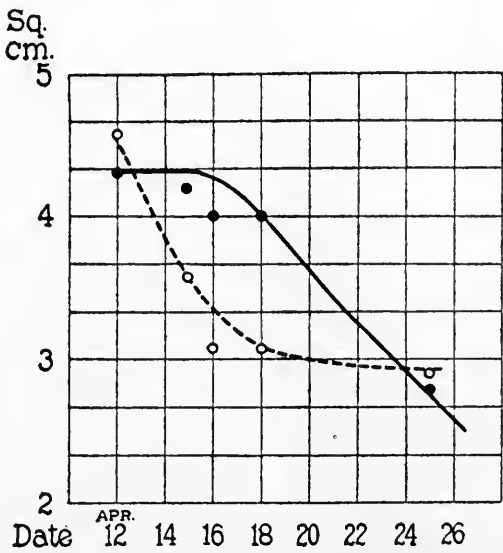
II.

*Effect of Irritants.*

In a second series of experiments, it was investigated whether the application of mild irritants on the surface of the wound would

TABLE II.  
*Action of Turpentine on the Latent Period.*

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
6	6	1921	sq. cm.			sq. cm.			
		Apr. 12	4.3		Chloramine-T.	4.6		Turpentine.	Text-fig. 4. No displacement of dressing.
		" 15	4.2	0	Dry gauze.	3.6	0	Dry gauze.	
		" 16	4.0	0	" "	3.1	0	" "	
		" 18	4.0	0	" "	3.1	0	Turpentine.	
		" 25	2.8	0		2.9	1		



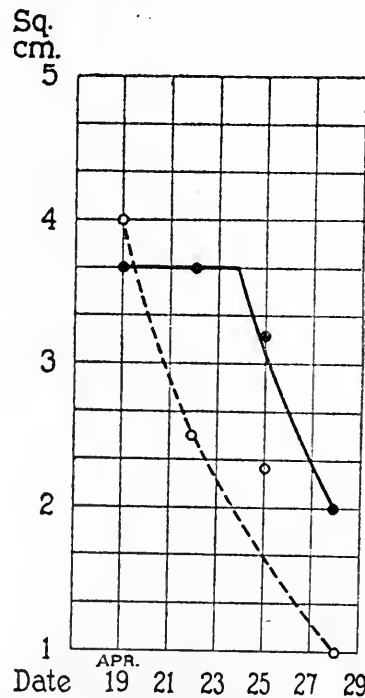
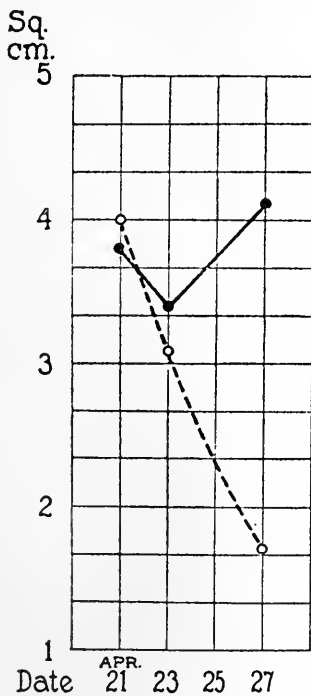
TEXT-FIG. 4. Experiment 6, Table II.

shorten the latent period. The experimental wound was covered by a gauze pad, soaked in turpentine and fixed to the edges of the skin by a few stitches, while the control wound was dressed with chloramine

paste. Both wounds were protected by gauze pads, sutured to the skin. The latent period of the experimental wound was very much shortened, lasting less than 2 days, while the latent period of

TABLE III.  
*Action of Chick Embryo Pulp on the Latent Period.*

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
7	7	1921	sq. cm.			sq. cm.			
		Apr. 21	3.8		Chloramine-T.	4.0		Chick embryo pulp.	Text-fig. 5.
		" 23	3.4	0	"	3.1	5	Dry gauze.	
		" 27	4.1	0	"	1.7	0	" "	



TEXT-FIG. 5. Experiment 7, Table III. TEXT-FIG. 6. Experiment 10, Table IV.

the control wound lasted for about 5 or 6 days (Table II and Text-fig. 4).

In Experiment 7, chick embryo pulp was used instead of turpentine (Table III). 6 days after the operation, the contraction of the

control wound had not started. On the contrary, in the experimental wound contraction began after a very short time, less than 2 days

TABLE IV.

*Action of Staphylococcic Infection on the Latent Period.*

Experiment No.	Animal No.	Date.	Control wound.			Experimental wound.			Remarks.
			Area.	Bacteria per field.	Dressing.	Area.	Bacteria per field.	Dressing.	
8	8	1921 Apr. 19	sq. cm. 5.0		Chloramine-T.	sq. cm. 4.0		1:100 dilution of staphylococcus suspension.	Mild infection; no edema; slight discharge.
		" 21		8	"		78	Dry gauze.	
		" 22	4.0	0	"	3.5	45	" "	
		" 25	3.2	0	"	3.3	24	" "	
		" 28	2.8	0	"	1.8	13	" "	
9	9	" 19	4.0		"	3.7		1:10 dilution of staphylococcus suspension.	Text-fig. 6.
		" 21		4	"		∞	Dry gauze.	
		" 22	3.7	0	"	3.4	85	" "	
		" 25	3.4	0	"	3.9	1	" "	
		" 28	2.8	0	"	1.7	20	" "	
10	10	" 19	3.7		"	4.0		1:50 dilution of staphylococcus suspension.	Text-fig. 6.
		" 22	3.7	0	"	2.5	50	Dry gauze.	
		" 25	3.2	0	"	2.3	0	" "	
		" 28	2.0	0	"	1.0	41	" "	
11	11	" 21	4.0		Chloramine-T.	3.5		Pure staphylococcus cultures.	
		" 23	2.6	0	"	2.4	6	Dry gauze.	
		" 25	2.5	0	"	2.2	0	"	
		" 28	2.3	0	"	0		"	

(Text-fig. 5). 6 days after the operation, the area of the experimental wound was about 50 per cent smaller than that of the control wound.

In four experiments (Table IV), the wounds were infected with staphylococci. Varied dilutions of a 24 hour culture of staphylococci in bouillon were used for inoculation. The control wound was dressed with chloramine paste, while the experimental wound was inoculated with 0.05 cc. of the dilution of staphylococcic culture, and dressed with dry gauze. The wounds remained in a condition of slight infection without swelling of the edges or abundant suppuration. The duration of the latent period was decreased, and often reduced to less than 2 days, as shown in Experiment 10 (Text-fig. 6).

### III.

#### SUMMARY.

As long as the wounds were protected by a connective tissue dressing against mechanical, chemical, and bacterial irritations, no evidence of cicatrization was found. The complete or partial failure of four experiments was due to the slipping of the inner dressing from the wound, mechanical irritation by the gauze, and infection. In the two experiments in which the connective tissue was maintained at the surface of the wound, there was no beginning of cicatrization, although 25 and 18 days respectively had elapsed since the operation, while in the control wound the duration of the latent period did not exceed 5 or 6 days. The experiments were interrupted after the second or third inspection, on account of the technical impossibility of again applying to the wounds a non-irritant dressing. It is probable that the wounds could have been kept for a much longer time in a condition of quiescence. While it is not known whether cicatrization could be prevented for an indefinite period, there is no doubt that the mechanism of regeneration is not set in motion at the usual time, when all external irritations are suppressed. It appears, therefore, that under ordinary conditions, cicatrization is not initiated by an internal factor.

On the contrary, the application of turpentine, chick embryo pulp, and staphylococci decreased markedly the length of the latent period, which was often reduced to less than 2 days. This fact demonstrated the importance of external factors in the initiation of cicatrization. It seems that the mechanism of regeneration has

become adapted to the ordinary conditions of life of the animals. A small wound will begin to cicatrize sooner if slightly infected, as practically always happens, than if it were thoroughly protected by a non-irritant dressing.

#### IV.

#### CONCLUSIONS.

1. It may be concluded that, under the conditions of the experiments, a wound, protected by a non-irritant dressing, shows no granulation tissue or beginning of contraction for 25 days at least.
2. Local application of certain irritants, such as turpentine, chick embryo pulp, and staphylococci, reduces the duration of the latent period to less than 2 days.
3. Regeneration is apparently initiated, not by an internal, but by an external factor.

# REMOTE RESULTS OF COMPLETE HOMOTRANSPLANTATION OF THE CORNEA.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 34.

(Received for publication, June 6, 1921.)

Homoplastic transplantation of the cornea in its entire thickness has been attempted many times with practically constant failure. However, if this operation be performed under proper conditions, the transplanted fragments can remain transparent, and the curvatures of the cornea normal. This fact was demonstrated by Zirm, who resected part of a leucoma and substituted for it a flap of normal cornea taken from the enucleated eye of a boy.<sup>1</sup> About a year after the operation, the patient could see through the transplanted cornea. Although it had been implanted in scar tissue, the flap had remained transparent. Such a result is exceptional, and many other attempts have been unsuccessful. As the inadequacy of the technique was probably responsible for the failures of the operation, it would be important to improve the method of transplanting the cornea in such a way that it could be used as a routine procedure in cases of leucoma. The purpose of the experiments described in this paper was to develop a better technique for homoplastic transplantations.

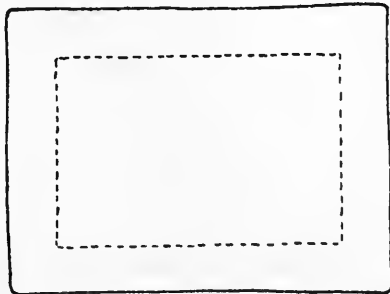
The technique consisted essentially in using a very large rectangular flap, fitted like the door of a safe into the edges of the corneal opening, and in fixing it securely in its position by stitches. This technique had been developed by Carrel in 1912, in the course of unpublished experiments on autotransplantation of the cornea. We attempted to adapt it to homoplastic transplantation, and to observe the results over a long period of time.

1. *Preparation of the Eye.*—The experiments were performed on cats. Cultures in bouillon were made of the secretion of the con-

<sup>1</sup> Zirm, E., *Arch. Ophth.*, 1906, lxiv, 580; *Wien. klin. Woch.*, 1907, xx, 61.

junctiva and only animals whose conjunctiva was found to be sterile were used. The hair covering the palpebra and the skin surrounding the eye were cut with scissors. Several days previous to the operation, the eye and the surrounding skin were washed with mercury bichloride at 1:5,000. Immediately before the operation, the surrounding skin and that of the palpebra were washed with alcohol, and afterwards tincture of iodine was applied.

2. *Preparation of the Graft.*—The graft was taken from the eye of a cat under ether anesthesia. The outline of a rectangular flap 6 by 8 mm. was traced on the surface of the cornea with a sharp cataract knife. The surface was incised to a depth corresponding to about half the cornea. Then the internal edge of the flap was dissected a distance of 1 mm. The anterior chamber was opened, the incision of the posterior part of the cornea continued with the scissors, the flap removed, and put in olive oil until it was used. Its anterior surface was 6 by 8 mm., while its posterior surface was only 4 by 6 mm. (Text-fig. 1).



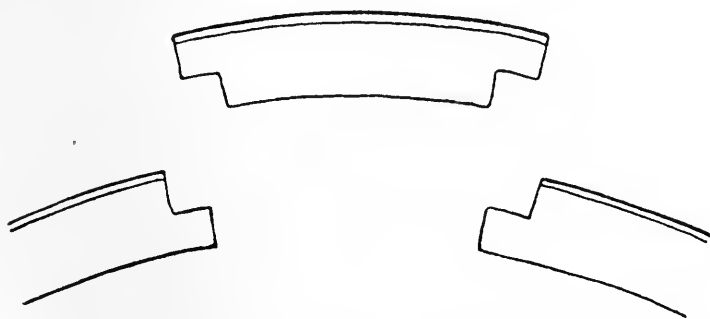
TEXT-FIG. 1. Anterior aspect of flap.

3. *Resection of the Cornea and Transplantation.*—The animal was etherized by the Meltzer-Auer method. The head was placed on a sand-bag and covered with a black silk towel, perforated in the center. The operating field was widely exposed by four small forceps fixed to the conjunctiva. The graft was placed on the surface of the cornea and the outline of the fragment to be resected was traced with the point of a cataract knife. Then the graft was replaced in olive oil. The incision of the cornea was made to a depth of about 1 mm. Then the internal edge was dissected for 1 mm., the anterior chamber was opened, and the fragment resected (Text-fig. 2). The graft was

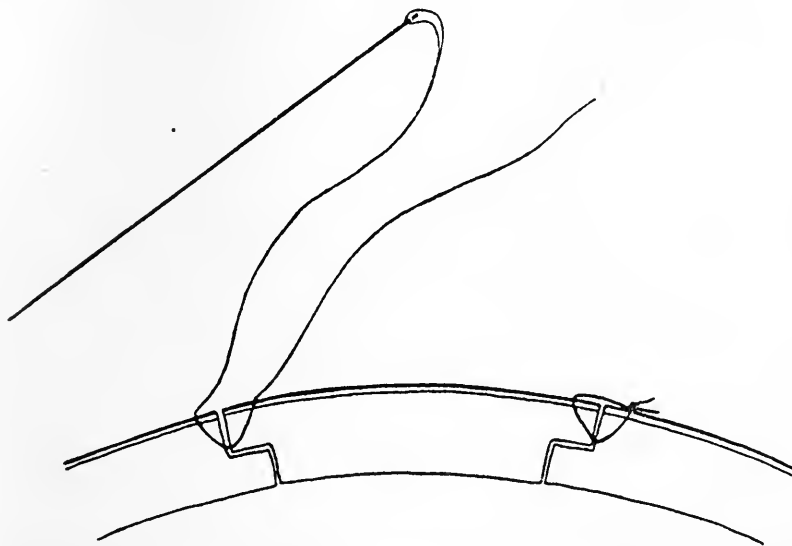


immediately inserted in the opening, where it fitted the edges of the cornea exactly (Text-fig. 3).

4. *Fixation of the Graft*.—The suture of the graft to the cornea was made with straight needles, No. 16, and silk sterilized in vaseline. As the corneal tissue is very hard, prong-toothed dissecting forceps



TEXT-FIG. 2. Cross-section of corneal flap and opening in the cornea, showing step edges.

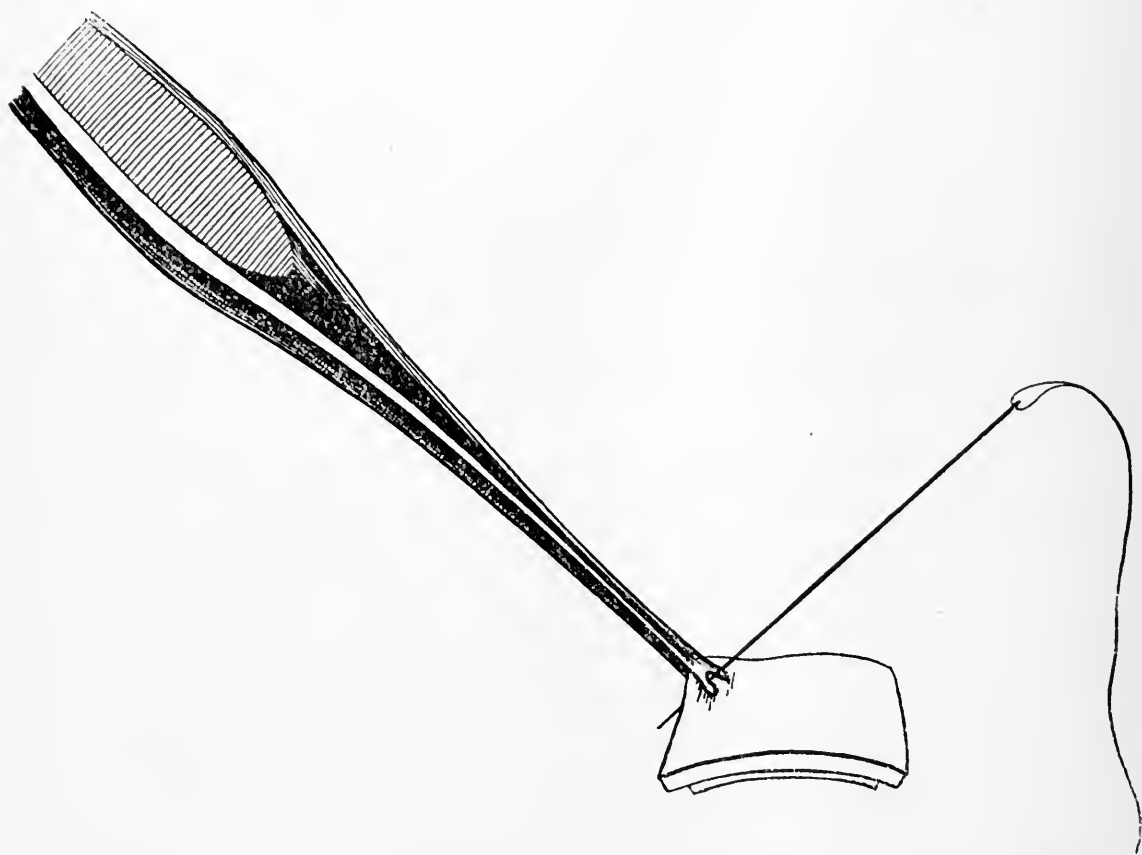


TEXT-FIG. 3. Transplanted flap in position, showing stitches passing through the superficial layers of the transplant and edge of opening in the cornea.

were used, the ends of which were bifurcated like a very small, double tined fork. The edge of the flap was seized by the forceps and the needle pushed through the tissue between the tines (Text-fig. 4). Only the superficial part of the cornea was caught by the needle. Each angle of the flap was fixed by one stitch. Another stitch was placed on the longer side of the rectangle. On account of the shape

of the edge, the incisions were closed tightly, and there was no leakage of fluid from the anterior chamber through the line of incision (Text-fig. 3).

5. *Postoperative Treatment.*—In previous experiments, it had been found that the dressing was often a cause of irritation. Therefore no dressing was applied. The animals kept the operated eye closed for several days after the transplantation. The corneal stitches fell out after a short time.



TEXT-FIG. 4. Prong-toothed dissecting forceps holding corneal flap and needle stuck through the tissue.

The experiments were made during an epidemic of distemper. Five cats were operated upon. Two died from distemper some time after the operations. Two others had a marked local infection of the eye and the cornea became completely opaque. In the fifth experiment (Cat 1, performed on May 20, 1919) the animal remained in good condition and the cornea transparent. A month later, the stitches had disappeared, the cicatrization was perfect, the cornea clear, and its curvatures did not appear to be modified. The iris was

not adherent to the cornea. There was a very small opaque spot near the inner canthus. Since that time, the animal has continued in the same condition. On May 26, 1921, the cornea was perfectly transparent, the only evidence of a previous operation being a hardly visible opaque spot on the internal part of the cornea (Fig. 1). An examination was made by Dr. W. B. Doherty, the results of which are as follows:

No line of demarcation to indicate the limitation of the corneal graft could be seen with a Zeiss loupe. Corneal epithelium perfectly even. No lack of corneal luster. Oblique illumination showed no inequalities of the corneal surface. Cornea was sensitive. Transplant perfectly transparent; no infiltration or vascularization. Ophthalmoscopic examination showed clear image of the fundus. Near the inner canthus and in the portion comprising the original cornea, there was a faint infiltration of the deeper layers with two or three small blood vessels. This opacity was of a striate appearance and gave the impression that it was produced by trauma during the operative procedure.

The result obtained in this experiment shows that the technique which was used allows a perfect reconstruction of the cornea, even when a very large fragment has been resected. The cutting of the flap is not difficult after a little training, and there is no doubt that the fixation of a flap fitted like the door of a safe to the step-edged cornea by suture increased the safety of the operation in a great measure. It is known that, in the previous attempts to transplant the entire thickness of the cornea, a disc of cornea was merely placed in an opening made with a trephine. In the operation performed by Zirm, the disc was maintained by two cross threads stitched to the conjunctiva. There is certainly a great advantage in cutting and fixing the flap in such a way that the anterior chamber is tightly closed, and that no displacement of the graft can take place.

#### SUMMARY.

1. A flap composed of the entire thickness of the cornea of a cat was transplanted to the cornea of another cat, and was found to be perfectly transparent 2 years after the operation.
2. The curvatures of the cornea appeared to be normal.

We wish to acknowledge our indebtedness to Dr. Doherty for the clinical examination of the eye and the report of the findings.

EXPLANATION OF PLATE 34.

FIG. 1. Cat 1. Right eye operated upon. Photograph taken May 31, 1921, 2 years and 11 days after the transplantation was made. The cornea appears normal. There is practically no difference between the cornea of the left eye and that of the right.



FIG. 1.

(Ebeling and Carrel: Complete homotransplantation of cornea.)



# REMOTE RESULTS OF OPERATIONS ON THE PULMONARY ORIFICE OF THE HEART.

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(Received for publication, June 6, 1921.)

In 1913, an attempt was made to develop a technique by which the size of the pulmonary orifice of the heart could be increased or decreased. The immediate results of the experiments showed that plastic operations on the wall of the pulmonary artery and its sigmoid valves caused little danger to the life of the animal, when a proper procedure was employed. The purpose of this paper is merely to examine the remote effects of the operations.

## I.

### EXPERIMENTAL.

In four experiments, the pulmonary orifice was enlarged by a vertical incision and suture of a flap of vessel.<sup>1,2</sup> In five others, the sigmoid valves were cauterized, incised, sutured, and the circumference of the orifice was shortened by stitching two of the valves together.<sup>2,3,4</sup>

1. *Patching of the Pulmonary Orifice.*—The operation consisted in suturing to the anterior side of the orifice a flap of a vein which permitted an increase in the circumference of the orifice after the wall had been incised.<sup>1,2</sup>

*Experiment 1.*—Black, female dog; medium sized, No. 1. October 23, 1913. The patch was a fragment of human aorta taken 24 hours previously from a cadaver and preserved in cold storage. The incision was made on the anterior side of the pulmonary artery. May, 1914. Animal still in normal condition. October 16. Died.

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<sup>1</sup> Tuffier, T., and Carrel, A., *J. Exp. Med.*, 1914, xx, 3.

<sup>2</sup> All operations were performed under ether anesthesia.

<sup>3</sup> Carrel, A., *J. Exp. Med.*, 1914, xx, 9.

<sup>4</sup> Carrel, A., *Ann. Surg.*, 1914, lx, 1.

*Autopsy.*—Adhesions of the lungs to the thoracic wall. Heart surrounded by adhesions. Adhesions of the pericardium. Anterior side of the pulmonary artery covered by a layer of fibrous tissue. Surface of the intima of the pulmonary artery smooth, and sigmoid valves entirely normal. Above the anterior valve, depression of the wall about 8 mm. wide, 18 mm. long, and 2 mm. deep.

*Experiment 2.*—Black and white bulldog, medium sized, No. 2. October 31, 1913. The patch was made of a flap of dog jugular vein, preserved for 24 hours in cold storage. May 20, 1914. Animal normal. March 3, 1915. Died after an illness of several weeks.

*Autopsy.*—Adhesions of the lung to the thoracic wall. Fluid in the pleural cavity. Adhesions of the pericardium. A thick layer of fibrous tissue on the anterior part of the pulmonary artery and the upper part of the ventricle. Indistinct and narrow scar above the anterior sigmoid valve of the pulmonary artery. No apparent depression of the wall. Surface of the intima smooth. Sigmoid valves normal. Location of the patch detected by thickening of the wall.

*Experiment 3.*—White, male bulldog, No. 3. November 26, 1913. Patching of the pulmonary artery with a piece of dog vena cava, preserved for 1 month in cold storage. May, 1914. Animal normal. February 17, 1915. Died.

*Autopsy.*—No adhesions of the pericardium. No adhesions of the pulmonary artery to the aorta or the auricle. Connective tissue on the anterior part of the vessel thickened. No scar visible from the outside. Opening of the vessel along the posterior side. Sigmoid valves normal. No apparent insufficiency. Slight depression of the wall above the anterior valve. Surface smooth and scar very indistinct. Upper part of the scar 1.5 cm. above the sigmoid and hardly visible. Location of the patch detected only by a slight thickening of the wall.

*Experiment 4.*—White, female bulldog, No. 4. December 2, 1913. The incision of the pulmonary artery was patched by a flap of jugular vein, preserved in cold storage since November 28, 1913. May, 1914. Animal in good condition. December 21. Died.

*Autopsy.*—No adhesions of the pleura or the pericardium. However, a few loose adhesions at the posterior part of the heart. Anterior part of the pulmonary artery free from fibrous tissue. No scar on the external side. After opening of the artery, an indistinct and slightly depressed scar visible above the anterior sigmoid. Surface of the intima smooth. Patch located by a slight thickening of the wall.

*2. Cauterization, Section, and Suture of the Sigmoid Valves.*—After longitudinal opening of the artery, just above the orifice, the sigmoid valves were cauterized with the thermocautery, or they were severed and sutured, or two of them were stitched together.<sup>2,3,4</sup>

*Experiment 5. Cauterization of the Sigmoid Valves.*—Young brindle, female bulldog, No. 5. April 14, 1914. Cauterization of the edges of the left and right



sigmoid valves with the thermocautery. June, 1921. Dog in good condition. No murmur.

*Experiment 6. Cauterization of the Sigmoid Valves.*—White and yellow, male fox-terrier, No. 6. April 29, 1914. Cauterization of the anterior and the right valves. December 29, 1916. Died.

*Autopsy.*—Adhesions of the lungs to the pericardium. Heart enlarged. Adhesions of the pericardium and the pulmonary artery to the aorta and the left auricle. Fibrous tissue on anterior face of the pulmonary artery. Anterior sigmoid valve rigid and thickened, especially along its margin. Left posterior valve normal. Two holes about the size of a pin-head near the anterior insertion of the right valve. Scar of the incision of the arterial wall hardly visible, especially in the center.

*Experiment 7. Cauterization of the Sigmoid Valves.*—White fox-terrier, with brindle spots, No. 7. April 30, 1914. Cauterization of the posterior valves. Animal remained in good condition for 6 years and died of double pleuropneumonia, May 31, 1920.

*Autopsy.*—No adhesions of the lungs to the thoracic wall. Adhesions of the lungs to the pericardium. Pus in both pleural cavities. Loose adhesions of the pericardium at the anterior part of the heart. Linear scar at site of pulmonary incision. Sigmoid valves normal; no thickening of the edges. A few small holes in the wall of the posterior sigmoids near the margin.

*Experiment 8. Section of a Sigmoid Valve.*—Black, long haired dog, No. 8. March 10, 1914. Section of the right posterior valve with the scissors. Animal died during the period of the War. Heart, preserved in formaldehyde, examined May, 1920. Adhesions of the pericardium. Valve slightly rigid. No union of the incision; thickening of the edges.

*Experiment 9. Stenosis of the Orifice by Stitching Both Posterior Valves.*—Black and white, long haired, male dog, No. 9. March 12, 1914. The right and left sigmoid valves were united by a stitch at a distance of about 3 mm. from their insertion. Dog remained in good condition. May 10, 1918. Died of pneumonia.

*Autopsy.*—Pericardial adhesions. Margin of both posterior valves slightly thickened. Scar tissue on the wall of the artery at the insertion of the valves. No union of the valves and, therefore, no stenosis of the orifice.

*Experiment 10. Section and Suture of the Right Sigmoid Valve.*—Yellow and white, male fox-terrier, about 7 years old, No. 10. March 17, 1914. Right sigmoid valve was completely sectioned in the middle from the margin to the insertion. Approximation of the cut edges by a stitch. May 20. Slight diastolic murmur. June 8, 1920. Animal in good condition. No murmur. June, 1921. Same condition.

## II.

## DISCUSSION AND SUMMARY.

Eight animals died from 1 to 6 years after the operation, from undetermined diseases, or from pneumonia. Two animals are still alive 7 years after the operation.

1. *Condition of the Pleural and Pericardial Cavities.*—In the first experiments, extensive pleural and pericardial adhesions were observed 1 year and more after the patching of the pulmonary artery. There was also a great deal of fibrous tissue between the pulmonary artery, the aorta, and the left auricle. In the other experiments, the adhesions of the lungs, pleura, and pericardium were less marked. This was due to some improvements in the technique of handling the viscera. At the time of the operations, it was hoped that no pleural or pericardial adhesions would occur. Great care was taken not to injure the endothelial surfaces by rough handling or by sponging. No blood was allowed to flow into the pleural cavity. The surface of the pericardium was protected by fine silk membranes. The pleural cavity was occluded by thick pads made of cotton and Japanese silk. It seemed that the serous surfaces were almost completely protected against infection and mechanical irritation. The occurrence of primary pleurisy and pericarditis was prevented by this technique. But the development of adhesions in several of the experiments shows that the procedures for the handling of the viscera should be perfected.

2. *Condition of the Arterial Wall.*—In the experiments in which the orifice was patched, a slight dilatation of the artery was observed. It was not possible to ascertain from the specimen preserved in formaldehyde whether or not there was an insufficiency of the valves. It is probable that there was no leakage, as in none of these cases could any diastolic murmur be heard 6 months after the operation. The only animal which presented clinical evidence of pulmonary insufficiency died during the War. The normal condition of the pulmonary orifice was due to the incision which did not extend far enough on the ventricle, and to the power of reintegration possessed by an organ which is not diseased. The cicatrization of the grafted flap was excellent. Its outline could not be seen on the external side of the wall. Even after opening the artery, the transplant could not be

located easily. However, in Experiment 1 the anterior wall of the artery showed a depression about 7 or 8 mm. wide, 18 mm. long, and 2 or 3 mm. deep, behind and above the anterior valve. But the flap was made of human artery, and it is known that a heteroplastic graft always undergoes some dilatation. When transplants of dog tissue were used, no dilatation occurred and the location of the patch could hardly be detected. In Experiment 7, 6 years after the operation, the endothelial surface was smooth, glistening, and no scar could be seen. However, the upper and lower parts of the incision were marked by a slight depression of the wall. The presence of the patch was detected by a distinct thickening of the wall. Although the edges of the incision had not been sutured to the edges of the flap, the endothelial surface was quite smooth. A transverse section of the artery was made through the middle part of the flap in Experiment 4. It showed the width of the arterial opening and the way in which the transplant became adherent to the arterial wall. The examination of these four specimens demonstrated that, in spite of the unfavorable location of the graft, an excellent union had taken place. It showed that homoplastic or heteroplastic tissue can be transplanted onto the pulmonary artery as well as onto the smaller arteries. Where the arterial wall had simply been incised without interposition of a patch, a linear scar was always found. 6 years after the operation, the incision used in the course of an operation for cauterization of the sigmoid and sutured with heavy thread was transformed into a linear scar and the surface of the intima was quite smooth.

3. *Condition of the Sigmoid Valves.*—In three experiments, the sigmoid valves had been cauterized along their margin and their point of insertion in the artery. One of the animals was still living 7 years after the operation. There was no diastolic murmur. The other animals died 3 and 6 years after the operation. The valves were thin and transparent, and quite normal. However, one of the valves showed two holes, one near the base and the other near the margin.

The animal on which the section of the right posterior valve without suture was performed, died 2 or 3 years after the operation. The

edges of the incision had not united. They were thickened and the whole valve was rigid. The surface was rough and irregular.

No permanent result was obtained by the union of two sigmoid valves by a stitch. There was no stenosis of the orifice, and no union of the valves 4 years after the operation. The stitch had disappeared. There was some scar tissue at the common point of insertion of the posterior valves, which were more rigid and showed thickened edges. 7 years after the section and suture of a sigmoid had been performed, the animal was still living and in good health. No diastolic murmur could be detected.

# GROWTH OF FIBROBLASTS AND HYDROGEN ION CONCENTRATION OF THE MEDIUM.

By ALBERT FISCHER, M.D.

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(Received for publication, June 6, 1921.)

The purpose of the experiments described in this paper was to determine the rôle played by the hydrogen ion concentration of the medium with regard to the growth of a strain of fibroblasts cultivated *in vitro* for a long period of time. The fibroblasts used were derived from fresh embryonic chick heart, from 1 or 2 months old strains of connective tissue, and from a strain cultivated for 9 years.<sup>1</sup> The cultures were prepared according to a technique previously described.<sup>2</sup> The method used for determining the hydrogen ion concentration has been described recently by Felton.<sup>3</sup>

## I.

### *Method of Obtaining Variations in the Hydrogen Ion Concentration of the Media.*

The variations in the hydrogen ion concentration of the media were obtained in the following ways.

1. *Acid Solutions.*—Solutions of acids were made in such concentrations that the addition of a small amount, usually 1 drop, to a certain amount of embryonic tissue juice or plasma would give the hydrogen ion concentration desired. A quantity of juice or plasma sufficient for a whole series of experiments was made in order to secure the same reaction in all. One of the disadvantages of this method was that plasma prepared in quantity underwent changes which resulted in decreasing or entirely preventing coagulation. A second disadvantage was that the embryonic tissue juice sometimes caused subsequent precipitation.

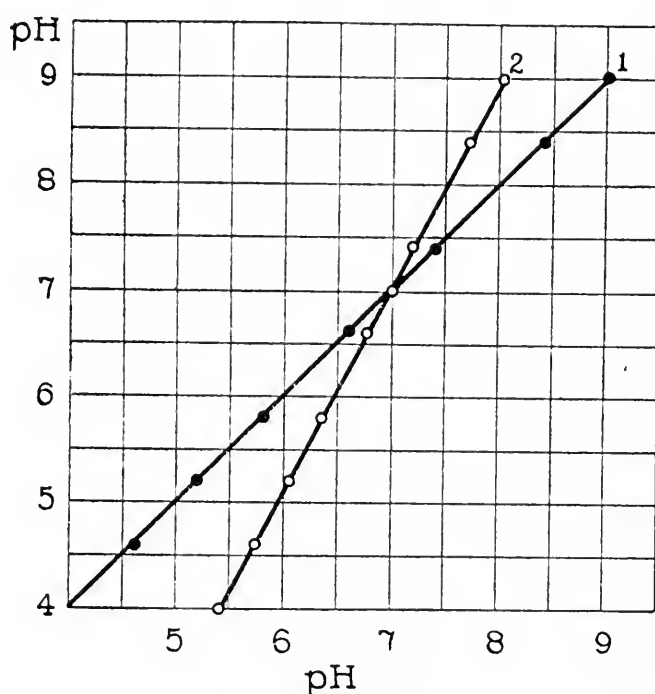
<sup>1</sup>Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

<sup>2</sup>Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531-535.

<sup>3</sup>Felton, L. D., *J. Biol. Chem.*, 1921, xlvj, 299.

2. *Phosphate Solutions.*—Sørensen's standard phosphate solutions were added to embryonic tissue juice in equal parts, to obtain different hydrogen ion concentrations in the media. It was found that if equal parts of fresh embryonic tissue juice and sterile buffer solutions were mixed, for instance with a pH of 7, the growth obtained was nearly as extensive as that in the control in which the juice was diluted with Ringer solution. This showed that phosphate had no appreciable influence on the rate of growth.

A known hydrogen ion concentration was obtained by the following technique. Some preliminary experiments were made in order to find



TEXT-FIG. 1. The abscissæ indicate the pH obtained, and the ordinates the pH of the buffer solution added. Curve 1 represents the pH of Ringer solution, and Curve 2 the pH of embryonic tissue juice, to both of which are added the different standardized buffers as indicated by the ordinates.

out the pH of a mixture of embryonic juice and a given acid. Different numbers of drops of the respective solutions (acid or buffer) were added to tubes holding the same amounts of juice, and the hydrogen ion concentration was tested. These different known reactions of the juice obtained in this way gave a curve in which the amount of acid necessary to obtain a certain hydrogen ion concentration between the

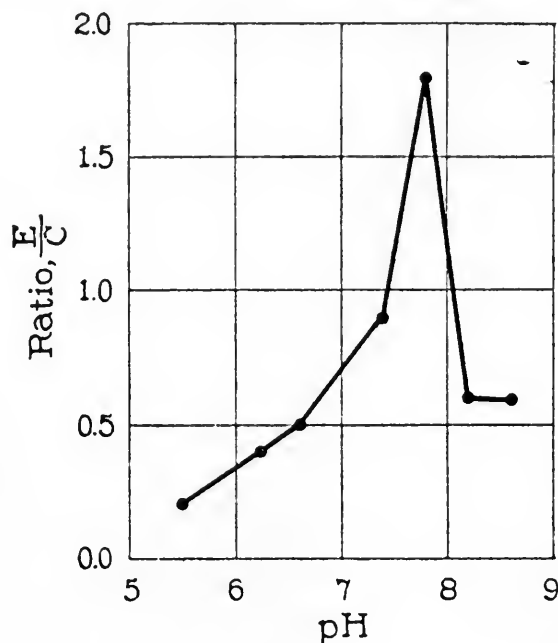
points found empirically could be calculated by interpolation (Text-fig. 1). Equal volumes of the standardized buffer solutions and of tissue juice were used. Upon ascertaining what pH resulted in the tissue juice when it was mixed with its own volume of buffer solution, the reactions obtained gave a straight line when a curve was plotted and it was possible to calculate readily which buffer should be used to produce the hydrogen ion concentration desired in the tissue juice (Text-fig. 1). Another test made in the same way was then necessary; namely, with the plasma-juice mixture. This had to be done rapidly in order to mix the indicator and juice-plasma before coagulation took place. In other words, when fibroblasts are to be cultivated in a medium of, let us say, pH 6, preliminary experiments for those particular media will show what buffer solutions should be added to the juice which will give pH 6, when combined with plasma. The test should be made in two steps for each experiment, one for the juice-buffer, and the other for the juice-buffer-plasma mixture, because of the slight differences in hydrogen ion concentration of the different juices and plasmas. The tissue juice may vary from pH 6.8 to 7.2, and the plasma from pH 7.4 to 8.

## II.

### *Cultivation.*

After the embryonic juice had been tested to determine its hydrogen ion concentration, it was mixed with equal parts of a buffer solution, calibrated pipettes being used to obtain the same sized drops. A selected culture was divided in two equal parts and washed in Ringer solution for about 30 seconds. One fragment was cultivated in a medium composed of 1 drop of plasma and 1 drop of a mixture of embryonic tissue juice and buffer solution. The other fragment, or the control, was cultivated in 1 drop of plasma and 1 drop of embryonic tissue juice to which had been added the same volume of Ringer solution instead of buffer solution. Mica cover-slips were used. 1 hour after the preparation of the culture, a drawing was made under the projectoscope. After 48 hours incubation, the outline of the new growth was drawn and calculations were made, by the method described by Ebeling.<sup>2</sup> When the purpose of the experiments was the study of the influence of the same hydrogen ion concentration for several genera-

tions, the culture was divided in two parts at each passage. One fragment was kept as control and the other placed in the experimental medium, care being taken to keep the other factors constant.



TEXT-FIG. 2. The different hydrogen ion concentrations of the media in which the fourth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth,  $\frac{E}{C}$ . The different hydrogen ion concentrations in the medium were obtained by adding phosphate buffer solutions to the extract.

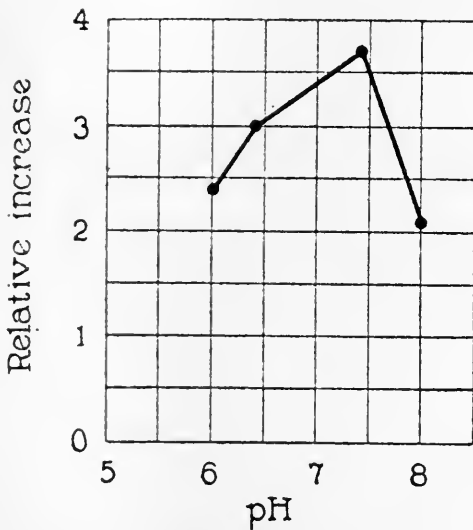
### III.

#### *Rate of Growth.*

When fibroblasts were cultivated in media containing different hydrogen ion concentrations varying from pH 5.5 to 8.5, a curve was obtained for the rate of growth with a distinct maximum between pH 7.4 and 7.8 (Text-figs. 2 to 4). The rate of growth decreased very rapidly with increasing hydrogen and hydroxyl ion concentrations, and the plotted curve was nearly symmetrical. The results obtained by using different acids, such as acetic, phosphoric, sulfuric, and hydrochloric, were much the same. It seemed that the anions did not have any remarkable influence on the growth. The curves reached a maximum at



pH 7.4 and fell very rapidly on both sides of this point, appearing always to be steeper on the alkaline side in spite of the fact that the resistance against the alkalinity was more marked. The same type of curve was found in all experiments. When the media were more alkaline or more acid, precipitation occurred, coagulation was interfered with, and it was impossible to draw any conclusions from the results. If the series of experiments were repeated by carrying the fibroblasts to a new medium containing the same hydrogen ion concentration as was used in the preliminary culture, it was observed that the absolute



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. The different hydrogen ion concentrations of the media in which the first passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

TEXT-FIG. 4. The different hydrogen ion concentrations of the media in which the fifth passage of the old strain of fibroblasts is cultivated are indicated by the abscissæ. The ordinates indicate the relative increase of growth. Phosphate buffer solutions were used.

TABLE I.

Experiment No.	Culture No.	Buffer solution added.*	Juice-buffer mixture.	First passage.			Second passage.			Third passage.			Fourth passage.		
				Control.	Experiment.	Ratio, $\frac{E}{C}$ .	Control.	Experiment.	Ratio, $\frac{E}{C}$ .	Control.	Experiment.	Ratio, $\frac{E}{C}$ .	Control.	Experiment.	Ratio, $\frac{E}{C}$ .
		pH	pH												
1	264	4.0	5.5	7.0	0.4	0.6	6.2	3.1	0.5	3.0	2.7	0.9	6.1	1.7	0.2
2	265	4.6	6.0	5.4	0.1	0.1	6.3	2.4	0.3	6.6	3.0	0.4	6.5	0.9	0.1
3	266	5.2	6.2	3.1	7.3	0.4	16.5	4.7	0.2	3.0	1.7	0.5	6.6	3.0	0.4
4	267	5.8	6.6	1.8	1.5	0.7	8.7	4.1	0.4	4.6	3.3	0.7	3.0	1.7	0.5
5	268	6.6	7.2	4.7	3.9	0.8	12.4	1.8	0.1	4.2	5.1	1.2	5.8	3.4	0.5
6	269	7.0	7.4	10.8	10.8	1.0	12.2	11.9	0.9	4.8	6.8	1.4	7.3	6.6	0.9
7	270	7.4	7.8	10.4	6.6	0.6	4.3	5.1	1.1	9.9	12.1	1.2	2.1	3.9	1.8
8	271	8.4	8.2	11.8	4.5	0.3	12.1	7.9	0.6	10.3	8.1	0.7	3.7	2.3	0.6
9	272	9.0	8.6	7.2	6.3	0.8	11.1	7.7	0.7	12.7	8.4	0.6	7.6	4.9	0.6

\* 1 cc. of buffer solution was added.

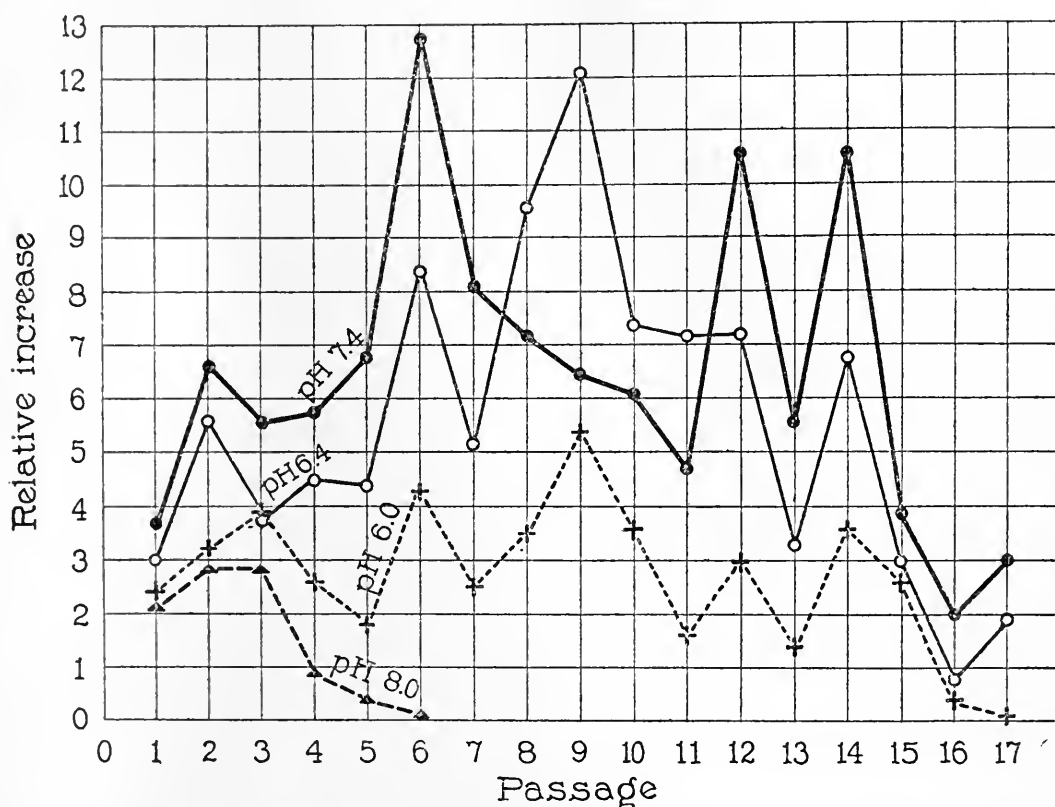
TABLE II.

Experiment No.	Culture No.	Buffer solution added.*	Juice-plasma mixture.	Relative increase.														
				Passage No.														
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		pH	pH															
1	375-1	4.0	6.0	2.4	3.2	3.9	2.6	1.8	4.3	2.5	3.5	5.4	3.6	1.6	3.0	1.4	3.6	2.6
2	375-2	5.8	6.4	3.0	5.6	3.7	4.5	4.3	8.3	5.2	9.6	12.1	7.3	7.2	7.2	3.3	6.8	3.0
3	375-3	7.0	7.4	3.7	6.6	5.6	5.8	6.8	12.7	8.1	7.2	6.5	6.1	4.7	10.6	5.6	10.6	3.9
4	375-4	9.0	8.0	2.1	2.8	2.8	0.9	0.4	0	0	0	0	0	0	0	0	0	0

\* 1 cc. of buffer solution was added.

increase of new growth became less and less in the most alkaline and most acid media (see also Tables I and II and Text-fig. 5). The descent of the curve on both sides of the maximum became more abrupt in the succeeding generations because of the retardation of growth in the media with highest alkalinity and acidity. (Compare Text-fig. 3, which represents the first passage, and Text-fig. 4, which represents

the fifth passage.) By following the growth of fibroblasts, generation after generation, in media containing the same hydrogen ion concentration it was observed that in the highest acidity (pH 5.5) cell proliferation ceased after four to six passages. The fibroblasts showed more resistance to higher alkalinity, but at the highest (pH 8.5) they grew for about eight to ten passages. It may be seen from the curve (Text-fig. 2) that the growth of tissue at pH 5.5 was very near the reaction where



TEXT-FIG. 5. The growth of the four cultures of the experiments in Table II, expressed graphically.

no growth will take place, although at pH 8.5 it was further from this point, and growth was observed for more than ten passages. It may be assumed, therefore, that growth can continue for a long time in this hydrogen ion concentration, but with a smaller increase of cell proliferation. The optimum growth occurred between pH 7 and 7.8. This was the normal reaction of a mixture of plasma and embryonic tissue juice. It may be seen from the curves that a slight variation in the hydrogen ion concentration of the culture medium resulted in marked changes in

the rate of growth of fibroblasts. It is interesting to note that the differences were mostly of a quantitative nature. The only morphological change of the cells observed was that, in the acid media, they showed more vacuoles than in the alkaline.

#### IV.

#### CONCLUSIONS.

1. The rate of growth of fibroblasts is markedly modified by slight changes in the hydrogen ion concentration of the medium. The curves expressing the rate of growth in function of the hydrogen ion concentration of the medium are nearly symmetrical on both sides of the maximum.

2. The optimum growth of fibroblasts occurs at pH 7.4 to 7.8. A slight change from this reaction has a remarkable influence on the rate of growth.

3. Fibroblasts show more resistance to higher alkalinity than to higher acidity. They grew for only four to six generations in a medium having a pH of 5.5, and for more than ten generations in one of 8.5.

4. The influence of different hydrogen ion concentrations on fibroblasts was only of a quantitative nature.

I wish to acknowledge my appreciation to Dr. Felton for his advice regarding his own method for measuring the hydrogen ion concentration of small amounts of fluid.

## STUDIES ON BACTERIAL NUTRITION.

### III. PLANT TISSUE, AS A SOURCE OF GROWTH ACCESSORY SUBSTANCES, IN THE CULTIVATION OF *BACILLUS INFLUENZÆ*.

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The preceding papers (1-3) in this series have dealt with the nature and action of the substances contained in blood which are of importance in the growth of *Bacillus influenzae*.

Search for substances other than the hemoglobin of warm blooded animals, which might be capable of stimulating growth of *B. influenzae*, has been made by several investigators. Davis (4) found that substances similar in their function to hemoglobin, namely hemocyanin, hemoerythrin echinochrom, did not sustain growth. He was unable to find any growth-inducing action in substances which readily give up oxygen, such as hydrogen peroxide and colloidal platinum, and among the various salts and organic preparations of iron he found none capable of replacing hemoglobin. Davis (5) has confirmed the observation of Ghon and von Preyss (6) that *B. influenzae* will grow on hematin agar only in the presence of dead or living bacteria. Davis points out that while hemoglobin in plain agar yields growth, still more abundant multiplication occurs when hemoglobin is associated with bacteria or tissues, either plant or animal, and especially if these are living. However, he states that plant and animal tissues, and bacteria by themselves will not sustain growth.

Olsen (7), studying the effect of blood on growth of Pfeiffer's bacillus, tested the growth-inducing action of various constituents of blood and derivatives of hemoglobin. He found that serum and ether extracts of washed blood corpuscles were ineffective. He observed, further, that hemoglobin and methemoglobin were capable of supporting growth, while hematin and hemin, on the other hand, were effective only in association with other bacteria. This author suggests that hemoglobin acts as a catalytic agent in rendering oxygen available for the bacilli.

In a recent publication on the nature of the effect of blood pigment upon the growth of *B. influenzae*, Fildes (8) favors the view that the iron-containing pigments function as catalysts in accelerating the transfer of oxygen to the bacilli. In interpreting the relative feebleness of growth on unchanged blood compared

to that on changed blood, Fildes attributes the growth differences to the fact that the oxygen affinity of unchanged hemoglobin may, by its own avidity, divert the oxygen from the bacilli, while physiologically inactive derivatives cannot intercept the oxygen liberated by the catalytic action of the iron in these pigments. In his experiments, however, Fildes never observed the occurrence of growth of *B. influenzae* in the total absence of blood pigment.

In preceding papers (1-3) the authors have pointed out that the hemophilic bacilli, of which Pfeiffer's bacillus serves as a type, require for their growth two distinct and separable substances, both of which are present in blood and neither of which alone suffices. These substances are (a) a vitamine-like substance which can be extracted from red blood corpuscles, and from yeast and vegetable cells, which is relatively heat-labile and absorbed from solution by certain agents; (b) a so called X substance which is also present in red blood cells, is heat-stable and acts in minute amounts.

The present paper concerns itself with the nature of this X substance in blood. It will be shown that this X factor can be derived also from sources other than blood, just as in a previous paper it was shown that the V factor can be supplied apart from animal tissue. That both the V and X factors exist in combination in nature will be shown by the fact that *Bacillus influenzae* will grow in blood-free medium provided certain plant tissues are added.

#### EXPERIMENTAL.

##### *Presence of the X Substance in Blood and Blood Derivatives.*

In a previous paper (3) it has been pointed out that the X factor, which constitutes one of the growth essentials of *Bacillus influenzae*, occurs in highest concentration in the cellular fraction of blood, and that the minimal effective amount is extremely small. In fact, the quantity of the X factor carried over in a loopful of the supernatant fluid of a blood broth culture of *Bacillus influenzae* is sufficient to induce growth in yeast extract broth; that is, in the presence of the supplementing vitamine-like substance, the V factor.

*Concentration of Accessory Substances in Blood Essential for Growth of Bacillus influenzae.*—From Table I, in which is shown the minimum concentration of hemoglobin permitting growth of *Bacillus influenzae* in broth with and without yeast extract, the relationship of the two factors, V and X, is again evident. While the source of the blood

in each instance is different, the two preparations of hemoglobin illustrate in a comparative way the minimal effective ratio between these two factors in the same preparation, and the extraordinarily minute quantity of the X substance which suffices for growth in the presence of an excess of the vitamine-like factor from yeast. The hemoglobin derived from laked blood cells was physiologically active; the crystalline hemoglobin had lost its oxygen-carrying capacity, although it still retained the characteristic absorption bands on spectroscopic examination. The latter preparation was devoid of

TABLE I.

*Concentrations of Hemoglobin Essential for Growth of B. influenzae.*

Concentration of hemoglobin in plain broth.*	Hemoglobin† from laked red blood cells.		Crystalline hemoglobin.‡	
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
1 : 10	++	++	—	++
1 : 100	++	++	—	++
1 : 1,000	++	++	—	++
1 : 10,000	+	++	—	++
1 : 100,000	—	++	—	++
1 : 1,000,000	—	+	—	++
1 : 2,000,000			—	+

\* All tubes containing 5 cc. total volume were inoculated with 0.05 cc. of yeast extract broth culture of *B. influenzae*. Plain broth without hemoglobin or yeast extract, and yeast extract broth alone served as controls and showed no growth under the same conditions of seeding.

† Hemoglobin from rabbit blood determined gasometrically by Van Slyke's method (Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127).

‡ Crystals of hemoglobin prepared from ox blood.

++ indicates marked growth; + moderate growth; — no growth.

the more labile vitamine-like substance as shown by the fact that of itself it was incapable of supporting growth even in the highest concentration. When complemented by the V factor in yeast extract, however, a 1:2,000,000 dilution of crystalline hemoglobin sufficed to stimulate growth of *Bacillus influenzae*. On the other hand, in the medium containing the solution of freshly laked blood cells both factors were present in amounts sufficient for growth up to a certain dilution, beyond which the native V factor was exhausted and the

presence of the residual X substance could be demonstrated only by adding the vitamine principle from yeast. Under the latter circumstances the X factor was still effective in concentrations of hemoglobin as small as 1:1,000,000.

Davis found that hemoglobin from human blood was active in promoting growth of *Bacillus influenzae* in dilutions as high as 1:180,000. Fildes (8) could not confirm the growth activity of sheep blood in these high dilutions. The sources of error suggested by Fildes, namely the carrying over of blood pigment from tube to tube, or with the inoculum from the culture itself, have been eliminated in the present study by the use of a separate pipette for each dilution, and by seeding all tubes with a small inoculum from a yeast extract broth culture known to contain only the minimal effective dose of the X substance. Furthermore, in the present experiments, broth rather than agar was used as the medium of choice. It seems not impossible, therefore, that these differences in methods, together with recognition of the need of the separation and titration of the two component factors individually, may account for the variations in question. Individual specimens of blood from the same or different species and corresponding derivatives of hemoglobin will of necessity vary in one or the other of these factors, particularly in their content of the V factor, so that comparisons of this sort express only relative values. Of more importance is the quantitative relationship, and the interdependence of these two factors in a given specimen, and the need, in testing for either substance in blood derivatives, of recognizing that the more labile vitamine-like factor may be destroyed in the preparation of the material tested, or lost by dilution without a corresponding destruction or loss of the X substance.

*Heat Stability of the X Substance in Blood.*—Sufficient data have been presented in the preceding papers of this series to establish the thermostability of the X substance. Unlike the V factor, the X substance in blood, blood extracts, and crystalline hemoglobin resists autoclaving at 120°C. for 45 minutes. Further evidence of the resistance of the X substance to heat is found in the fact that blood charcoal, which reacts positively to the benzidine test, retains the ability to function as the X factor in 10 per cent yeast extract broth; that is, to support growth of Pfeiffer's bacillus in the presence of the supplementing V factor.



*Benzidine Reaction.*—Olsen (7) states that both the guaiac and benzidine reactions go hand in hand with the ability of blood derivatives to support growth of *Bacillus influenzae*. This color reaction for the demonstration of the so called peroxidases has been found to parallel the presence of the X substance in blood. The question as to whether the positive benzidine-reacting substances and the X substance in blood are one and the same will be discussed later in describing the occurrence of these substances in plant tissue. It need only be remarked here, that in blood at least, the benzidine-reacting substances exhibit a marked resistance to heat, and in this instance again parallel the behavior of the X substance.

TABLE II.

*Absorption by Bone Charcoal of the X Substance in Crystalline Hemoglobin.*

10 per cent yeast extract broth 5 cc.* plus	Before absorption.		After absorption.	
	Benzidine test.	Growth-inducing action.	Benzidine test.	Growth-inducing action.
Crystalline hemoglobin, 1 : 1,000.....	++	++	—	—
Bone charcoal.....	—	—	++	++

\* Inoculated with 0.05 cc. of 18 hour yeast extract broth culture of *B. influenzae*.  
++ indicates positive benzidine test or marked growth.

*Absorption of the X Substance.*—It has been noted in the preceding paper of this series that the V factor in yeast extract can be completely removed from solution by absorption with bone charcoal. Similarly it has been found that the X substance can be absorbed from solutions of crystalline hemoglobin by this agent. The solution of hemoglobin after absorption no longer reacts positively to the benzidine test, and has suffered loss of the X substance as evidenced by the lack of any growth-inducing property.

From Table II it is evident that bone charcoal can absorb the X substance from solutions of crystalline hemoglobin. Absorption is facilitated by heat, and is related to the concentration of the X substance in solution and to the time allowed for the reaction. It is of interest to observe that the original bone charcoal failed to give the benzidine test, but, after absorption, reacted positively and had

itself acquired the X substance, as shown by its growth-promoting action in 10 per cent yeast extract broth. The supernatant solution of hemoglobin after absorption had lost both its benzidine-reacting and growth-promoting properties as shown in Table II.

In view of these facts, an attempt was made to determine whether the X substance present in blood might also be found in other proteins of animal origin, such as crystalline egg albumin, or in protein split products such as peptone, erepton, and "aminoids." None of these substances, however, gave a positive benzidine test and none could be substituted for the X factor in supplying the cultural needs of this organism. In these experiments the V factor was always supplied by the addition of yeast extract to the medium, so that if the X factor were present in any test substance, the growth requirements would be complete. Furthermore, lipoidal extracts of heart muscle with and without cholesterol, as described by Noguchi, were tried under similar conditions without result.

*Occurrence of the Growth Accessory Substances, X and V, in Plant Tissue.*

In the course of these experiments it was observed, as already noted, that a striking parallelism seemed to exist between the presence of the benzidine reaction in blood derivatives and the ability of these same substances to promote growth of *Bacillus influenzae*. This fact is not final evidence that the reacting substances are necessarily the same in both instances, but suggests rather that this color reaction for the so called peroxidases may serve as an indicator of the presence of the X factor in tissues other than those of animal origin. In the search for the X substance in vegetables, the potato was selected first, since it is known to contain peroxidases and catalysts and also both the fat-soluble A and the water-soluble B vitamins. For these reasons it was thought possible that raw potato would furnish both the V factor and the X substance. This was found to be the case by the luxuriant and continued growth of the bacilli which occurs in blood-free medium containing pieces of sterile raw potato.

*Technique.*—While no special attempt has been made to devise a precise method for obtaining pieces of sterile potato, and while

modifications will naturally suggest themselves to those trained in bacteriological technique, the following procedure is the one originally adopted. It is desirable to select a potato without surface abrasions or imperfections, and for this reason an old potato is preferable because of the thicker skin protection. Possibly differences in the content of the vitamines and the X substances in new and old potatoes exist, but these have not been determined. After thorough cleansing of the outer surface in running water, the skin is dried and well charred with a red-hot searing iron in a broad band encircling the potato. Through this seared band the potato is cut or broken open and from the inner portions small pieces are removed with a sterile scalpel and placed in sterile petri dishes, care being taken to avoid touching the margins or piercing the outer surface of the potato. The sections removed in this fashion are divided into pieces of suitable size and dropped into tubes of plain broth.

*Oxidizing Enzymes of Potato.*—It is well known that in plant, as well as in animal tissues, substances concerned with physiological oxidation and reduction are widely distributed. Scrapings from fresh potato exposed to the air rapidly change color as a result of oxidation processes. The benzidine reagent in the presence of hydrogen peroxide gives a blue color when applied to the cut surface of potato, the so called peroxidase reaction. Hydrogen peroxide alone applied in a similar way is rapidly decomposed with the liberation of gaseous oxygen through the action of a catalase in the potato. In studying the oxidase, peroxidase, and catalase of potato and other vegetables, Falk, McGuire, and Blount (9) noted that the enzyme reactions were destroyed by heating to boiling for several minutes. They observed that there was no well defined hydrogen ion concentration for maximum action of the vegetable enzymes, but that on the average the optimal zone lay between pH 7 and 10. According to the work of Cohn, Gross, and Johnson (10) on the isoelectric points of the proteins in certain vegetable juices, the natural hydrogen ion concentration of potato is pH 6 to 7.

Sterile raw potato possesses the property of slowly reducing methylene blue in solutions of phosphates ( $\frac{M}{15}$ , pH 7.5) from which atmospheric oxygen has been excluded by a vaseline seal. It has been noted

that, in hemoglobin, blood, or yeast extract broth under aerobic conditions, growth appears first in the upper layers of the medium where the oxygen tension is greatest. On the other hand, in broth containing raw potato under seal, growth of *Bacillus influenzae* occurs early in the depths of the medium about the vegetable. Although the natural reaction of potato is acid (pH 6 to 7), and despite the presence of active enzymes, the use of raw potato in well buffered broth (pH 7.8) does not necessitate further adjustment of the reaction for growth of *Bacillus influenzae*. In the cultivation of organisms more sensitive to reaction changes, the acidity developing in potato medium requires readjustment.

TABLE III.

*Effect of Heat on the Growth Accessory Substances of Potato.*

Inoculum, 0.05 cc. of yeast extract broth culture of <i>B. influenzae</i> .	Potato in plain broth.				Controls; plain broth.	
	Unheated.		Autoclaved at 120°C. for 45 min.			
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
Type* A.....	++	++	—	++	—	—
" B.....	++	++	—	++	—	—
" C.....	++	++	—	++	—	—
" D.....	++	++	—	++	—	—

\* Types A, B, C, and D refer to the biological classification of Stillman and Bourn (Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665).

++ indicates marked growth; — no growth.

The nature and interaction of these vegetable enzymes are too complex and too little understood to warrant any interpretation of their possible significance in the growth of *Bacillus influenzae* in media containing raw potato. The assumption that they may function as catalysts in facilitating the transfer of oxygen to the bacilli is perhaps justified by the fact that in potato broth, under vaseline seal, growth occurs in the depths of the culture about the vegetable tissue.

*Heat Stability of the Accessory Substances in Potato.*—From Table III it is evident that potato contains both the X and V factors requisite for growth of *Bacillus influenzae*, since small pieces of the fresh sterile

vegetable in plain broth suffice to stimulate rapid multiplication. Under these conditions, growth is not conditioned by the carrying over of either factor with the inoculum. Repeated experiments have amply confirmed these observations, and their validity is further established by the fact that growth in unheated potato broth is not limited merely to the first transfer, as is the case in yeast extract broth, but may be continued by loop inoculation from tube to tube.

In studying the effect of heat on the growth accessory substances of potato the interesting fact is brought out that the two factors, X and V, react to temperature in the same manner as the similar substances in blood. The vitamine-like principle is less resistant to heat than the X substance (Table III). Potato broth exposed in the autoclave to a temperature of 120°C. for 45 minutes is no longer capable of supporting growth of *Bacillus influenzae*. That the V factor is destroyed in the heating and that the more resistant X substance is left unimpaired is shown by the fact that the autoclaved medium can be reactivated by the addition of fresh active yeast extract.

Further evidence that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood for its peculiar nutritive requirements is afforded by the fact that *Bacillus influenzae* will grow in the complete absence of blood derivatives, meat extractives, and animal peptones. The addition of sterile raw potato to Uschinsky's synthetic medium containing asparagine and ammonium lactate suffices to support growth of *Bacillus influenzae*. A simpler medium of unheated potato in plain buffer solutions of sodium and potassium phosphate ( $\frac{M}{15}$ , pH 7.5) fulfills the necessary growth requirements of this organism. It is evident then that potato contains both the V and X substances and that these factors together with the native protein and carbohydrate of potato can replace in media blood pigment and tissue derivatives from animal sources.

In the absence of knowledge of the chemical nature of the X substance and in view of the chemical complexity of the tissues in which it occurs, the identity of the X factor must remain a matter of more or less conjecture. In blood, this substance seems to be associated with the iron-containing pigment, but attempts to substitute inorganic, organic, and colloidal forms of iron have been unsuccessful. The

occurrence of the X factor in potato, as well as in blood, suggests that this substance, or substances reacting similarly, may be commonly present in plant and animal tissue. Other vegetables have not been tested; banana, however, has been used and found to contain the essential growth factors. It is interesting to note that although furnishing the necessary growth accessory substances, banana fails to react positively to the benzidine tests. It possesses, however, a markedly active catalase, as evidenced by the evolution of gas when hydrogen peroxide is applied to the cut surface.

#### DISCUSSION.

The importance in animal nutrition of the presence in foodstuffs of growth accessory substances is now fully appreciated. There is a growing realization among biologists that this peculiar sensitiveness to the want of some particular substance in small amounts is not limited merely to the higher animals. In microbiology this principle finds its earliest expression in the work of Wildiers (11) (1901) on "bios," a substance extractable from yeast cells which exerts an accelerating influence upon the growth of yeast. Bottomley (12) has extracted from decomposing peat, substances which stimulate plant growth, and to which he has given the name "auximones."

The fact is also gaining recognition that bacteria require for growth not merely carbohydrates, proteins, and their derivatives suitably combined in a medium of optimal reaction. Many of the more difficultly cultivable microorganisms are sensitive to the lack in ordinary media of growth accessory substances allied perhaps to those of animal diet. The application of this principle to culture media has given rise to various modifications in the methods of preparation, each of which seeks to preserve or add certain substances, the presence or absence of which is recognized only by the growth-promoting value of the medium for a particular species. No group of organisms exhibits a more striking sensitiveness in this regard than the so called hemophilic variety.

The observations of Thjötta (2) on the multiplication of Pfeiffer's bacillus in hemoglobin-free medium have demonstrated that substances of bacterial origin can replace the growth-inducing factors of blood. It has been pointed out (1, 3) that there are two distinct and separable

substances in blood essential to the growth of this bacillus—a vitamine-like principle, the so called V factor, and a second substance, the so called X factor. Both of these substances are found in highest concentration in the cellular fraction of blood. They are both requisite for growth of hemophilic bacilli, and each is separately inactive and without effect. They differ in character one from the other in ways suggestive of the nature of their separate functions. The vitamine-like substance, the so called V factor, can be extracted from red blood corpuscles, from yeast and vegetable cells; it is relatively heat-labile, readily absorbed from solution by charcoal, required in greater concentration than the X factor, and resembles the known vitamins in its growth-promoting action. The second substance, the so called X factor in blood, is heat-stable, present in greatest concentration in red blood cells, absorbed from solution by charcoal, and effective in such minute amounts as to suggest a catalytic nature.

This dual function of blood in fulfilling the growth requirements of *Bacillus influenzae* has been appreciated though less fully analyzed by previous observers. Davis (5), particularly, has directed attention to the fact that for maximum development of hemophilic bacteria both pure hemoglobin and a second vitamine-like substance are essential. He suggests that these two principles in blood may be analogous to the fat-soluble A and water-soluble B vitamins. In the separation of blood pigment from the other constituents of peptic digests of blood, Fildes (8) observed that neither the clear, colorless filtrate nor the precipitated pigment alone could support growth of *Bacillus influenzae*. Combination of these two factors in media, however, afforded conditions favorable to growth.

*Bacillus influenzae* has heretofore been considered an obligate hemophile. The present studies indicate, however, that the hemophilic property of this group of organisms has been based on a lack of knowledge of their essential nutritional needs. Evidence is presented in this paper that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood or blood derivatives for its growth requirements. It has been shown that this organism will grow in the total absence of blood derivatives, meat extractives, and animal peptones. The growth accessory substances which occur in blood have been found to exist also in plant tissue. Sterile raw potato can replace blood in the cultivation of *Bacillus influenzae*.



On the basis of the facts presented, it seems not unreasonable to assume that nutritional deficiency in the cultivation of other bacteria may be overcome by the addition to culture media of the appropriate growth accessory substances.

#### CONCLUSIONS.

1. Growth of *Bacillus influenzae* depends on two distinct and separable substances.
2. These growth accessory substances are present in blood.
3. They occur in plant as well as in animal tissue.
4. Sterile, raw potato will serve as a substitute for blood in the cultivation of *Bacillus influenzae*.

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# STUDIES ON THE PHENOMENON OF D'HÉRELLE WITH BACILLUS DYSENTERIÆ.

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PLATES 35 TO 39.

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The phenomenon of d'Hérelle (1) is the expression of a lytic reaction occurring between a bacterium which is inducing an infection in an animal and a substance elaborated in that organism, probably by the leucocytes and other tissue cells, in response to the stimulus of the metabolic products of the invading bacterium. The important element of the reaction is that bacteria exposed to the lytic substance acquire the ability to transfer the lytic property to subsequent generations. The first observations of d'Hérelle were made on stool filtrates from patients convalescent from bacillary dysentery (1). As a result of this and further studies, d'Hérelle (2) concluded that whenever an animal offers resistance to a pathogenic bacterium, a bacteriophage, active for that bacterium, can be isolated from the dejections of the animal. He believes that defense by bacteriophage is a specific immunity reaction. Dumas (3) was able to demonstrate that filtrates from specimens of Paris city water, of Seine water, and of earth, were lytic for colon and dysentery bacilli. He consequently expressed the opinion that the phenomenon is non-specific. The explanation of Dumas' results, as McLeod (4) has stated, is that the specimens he examined were contaminated with excreta infected with colon or dysentery bacilli or both. Specimens from other places may give negative results.

## EXPERIMENTAL.

*Intestinal Contents.*

In an attempt to demonstrate d'Hérelle's phenomenon in human intestinal contents, twenty-three patients at the Babies' Hospital were examined. Six of the children gave a history of gastrointestinal disturbance, while one had been observed throughout an attack of Shiga bacillus dysentery during which the bacillus had been isolated from the stools. Specimens from twenty-two of the twenty-three patients, including the dysentery convalescent, when grown in broth for 24 hours and then filtered through a Berkefeld candle, showed only a negative (non-lytic) reaction between the sterile filtrate and young cultures of *Bacillus dysenteriae*. One positive reaction was obtained with the filtrate from the feces of an infant who had died of peritonitis due to *Bacillus coli* after intussusception of 3 days duration. In this instance the lytic action was stronger on the colon bacillus than on the dysentery bacillus, and it was possible to transfer the lytic quality to later generations of *Bacillus coli*.

The negative results obtained in the case of the infant who had recovered from an attack of Shiga dysentery may mean that the lytic quality of the intestinal contents is of short duration after such an infection. That the phenomenon of d'Hérelle is not a widespread one among children not ill of gastrointestinal disease is quite evidently demonstrated by these observations.

Dumas' (3) positive lytic reaction with the stools of five of eight adults who gave no history of previous intestinal infection would seem to show that there may be a difference between children and adults in this respect.

*Guinea Pig Peritoneal Exudate.*

Bordet (5) interpreted the lytic phenomenon of d'Hérelle as due to leucocytic action. He demonstrated its presence in the peritoneal exudate of guinea pigs inoculated with *Bacillus coli* on three occasions at intervals of 5 days. Repeated attempts to reproduce Bordet's results were negative, until a guinea pig was killed after only one intraperitoneal injection; that is, before the peritoneal exudate of the animal had become antilytic by repeated inoculations (Bordet).

The peritoneal exudate is withdrawn by means of sterile capillary tubes from the living animal, and diluted with twice its quantity of sterile broth adjusted to a pH of 8. This is in accordance with Gratia's (6) observation that the lytic action of d'Hérelle proceeds more readily and completely in an alkaline than in an acid medium. In order to rid the guinea pig exudate of fibrin it is well to draw it into a tube containing small sterile beads. The tube containing the defibrinated exudate is sealed and kept for 3 to 30 days to complete the leucocytic action (Bordet). At the end of that time it is centrifuged and the clear supernatant fluid heated for  $\frac{1}{2}$  hour at 58°C., after which it is ready to be tested on broth or agar slant cultures. Both must be young, not over 2 to 3 hours old. 1 drop of the fluid to be tested is dropped along one side of an inoculated agar slope, and the tube set upright in the thermostat for observation during the next 48 hours. To broth cultures a larger quantity of the fluid must at first be added, but after one or two generations the lytic fluid may be found to be active in high dilutions.

Growth may be prevented in a broth culture if the lytic agent be added when the tube is inoculated, or young bacilli may be dissolved if the fluid be added after the culture has grown 2 to 4 hours. A broth culture to which the lytic fluid has been added remains or becomes more or less clear for 12 to 24 hours, then it may become somewhat turbid, and clear again after another day. Even the clear tubes are not always sterile, as can be shown by plating. On agar slants the action of the lytic fluid is evidenced by a clear streak along the track of the fluid (Fig. 1). In this clear area there may be no colonies, or there may be a few; that is, some resistant bacilli may remain undissolved and viable. The action of the lytic fluid serves to separate a given culture into sensitive and resistant strains, and by repeating the action on the resistant strains a more and most resistant one can be obtained.

By filtering the clear fluid resulting from the action of the original lytic filtrate or exudate on a broth culture, any quantity of lytic fluid can be obtained, and its action on any number and variety of organisms tested.

The bacteriophage obtained by inoculating a strain of Shiga dysentery bacilli into a guinea pig was found to dissolve its homologous strain, five other Shiga bacillus strains, and two Flexner dysentery bacillus strains. On three strains of Hiss Y bacilli, however, its action was less marked; while three strains of colon bacilli, and one strain

each of typhoid, paratyphoid A and B, mouse typhoid, Morgan bacillus, hog-cholera, and fowl-cholera were not attacked.

Dr. Gratia kindly gave me a small quantity of a lytic fluid which he had prepared by allowing guinea pig exudate, obtained by Professor Bordet in Brussels, to act on a resistant strain of *Bacillus coli*. This bacteriophage proved to be strongly lytic for strains of *Bacillus dysenteriae* Shiga and less lytic for two strains of Flexner and three strains of Hiss Y dysentery bacilli. The Brussels strain of *Bacillus coli* as well as a strain isolated at the Babies' Hospital some months ago were readily dissolved, but a recently isolated strain of colon bacilli was not affected. *Bacillus typhosus* was acted upon to a slight extent only, but neither a paratyphoid A nor B strain was attacked.

These results showed that the lytic power of the colon phage, made with a resistant *Bacillus coli* strain, is not specific, but that it acts in varying degree on the related strains of the colon-typhoid-dysentery groups of bacilli. The observations of Bordet and Ciuca (7) announcing this fact were published just as our experiments were completed.

One strain each of Friedländer's bacillus, *Bacillus cholerae suis*, *Bacillus avisepticus*, *Bacillus typhi murium*, and Morgan's bacillus was tested with negative results.

With the purpose of studying more fully the effect of the action of a bacteriophage on the dysentery bacillus, a strain of the Shiga type was selected and exposed to the action of the colon phage. A new bacteriophage resulted which was strongly lytic for all strains of dysentery bacilli, for typhoid bacilli, and for the Belgian and American strains of *Bacillus coli*. The paratyphoid strains were still unaffected.

The anti-colon bacillus bacteriophage showed certain group reactions on members of the dysentery-colon-typhoid group. It was most active on the colon strain with which it had been made, less active on a heterologous colon strain; equally active on Shiga dysentery strains, less so on Flexner strains, and still less so on Hiss Y strains; and it was only slightly active on a strain of typhoid bacilli. The Shiga antidysentery bacillus phage, on the other hand, was completely lytic on all strains of Shiga dysentery bacilli and only slightly less so on all strains of Flexner and Hiss Y strains. It was completely lytic for Belgian and American strains of *Bacillus coli* and decidedly lytic for a typhoid bacillus strain. Its group reactions

were stronger than those of the colon bacillus phage. The question naturally presented itself as to whether this denotes a stronger bond between typhoid and dysentery bacilli than between dysentery and colon bacilli, or whether the lytic power of the dysentery bacteriophage was fundamentally stronger for all strains of these three groups. Weight is given to the former supposition by the fact that while the colon phage acted very slightly on the typhoid bacillus, a typhoid phage made from the colon phage by exposure to a strain of *Bacillus typhosus* acted much more strongly on dysentery than on *coli* strains.

Further study of the effect of the action of a lytic fluid upon a strain of Shiga dysentery bacilli is of interest. Not all the bacilli are dissolved, and it follows that those which survive are resistant to the lytic principle. Plates poured from a fluid culture of Shiga dysentery bacilli after 24 or 48 hours contact with a lytic fluid, when the broth culture tube appears to be perfectly clear, show two kinds of colonies (Figs. 2 to 4). One is round, with regular edges, a heaped up or denser center, and rather moist growth. The second kind is very irregular in size and shape, with deep indentations in its thin edges, a depressed center, and very granular. In broth the regular colonies grow rapidly and profusely, the broth becoming turbid. The irregular colonies grow more slowly and fall to the bottom of the tube in granular masses, leaving the broth slightly turbid or almost clear.

A strain of Flexner dysentery bacilli exposed to dysentery bacillus bacteriophage gave rise to regular and irregular colonies exactly comparable to those obtained from the acid or Shiga dysentery strains. Kuttner (8) has shown that the typhoid bacillus develops two kinds of colonies after action of a typhoid bacteriophage.

The experiment presented in Table I was done in order to test the reaction of the bacilli in the two kinds of colonies to the lytic fluid. The table shows that the bacilli in the regular colonies are more resistant to the lytic principle than are those in the irregular colonies.

To ascertain which colonies carry the lytic agent, *i.e.* which are lysogenic, the experiment presented in Table II was done. The irregular colonies are found, then, to be lysogenic and sensitive, while the regular colonies are non-lysogenic and resistant.

Both the regular and the irregular colonies were subcultured on plates for more than 40 generations, and Figs. 5 and 6 show the results. The regular colonies retain their regular outline and characteristics. The irregular colonies, on the other hand, gradually lose their markedly irregular outline and tend to approximate more nearly the regular type of colony, until there are more regular than irregular ones present, and even these are less radically irregular. That is, while the edges of the irregular colonies may still be deeply serrated, the centers are

TABLE I.

*Shiga Dysentery Bacilli Plus Shiga Bacteriophage.*

Strain.	Medium.	Age.	Result.	
			After 24 hrs.	After 48 hrs.
		<i>hrs.</i>		
Normal, or original.....	Broth.	2	Lysis; tube clear.	Same.
Regular.....	"	2	No lysis; tube turbid.	"
Irregular.....	"	2	Lysis; tube clear.	"

TABLE II.

*Normal or Original Strain of Shiga Dysentery Bacillus.*

Phage.	Medium.	Age.	Result.
		<i>hrs.</i>	
Normal, original bacillus filtrate.....	Broth.	2	Lysis.
Regular colony filtrate.....	"	2	No lysis.
Irregular " " .....	"	2	Complete lysis.

less hollowed out and gradually become dense (Figs. 7 and 8). The explanation seems to be that the sensitive bacilli die off more rapidly than the resistant ones, which form the regular colonies in later generations. It is a matter of selection.

The two kinds of dysentery bacilli, resistant and sensitive, do not differ from the original culture in their sugar reactions.

An attempt was made to determine whether the acquired lysogenic property is retained permanently or lost in later generations. Working with the original, normal culture as a whole, it was found that

after the seventh generation on agar or in broth the bacilli which had survived contact with a lytic fluid were no longer able to transmit the lysogenic property to other cultures or to dissolve the normal Shiga bacilli. Apparently the sensitive, lysogenic bacilli had all been eliminated in seven generations.

The experiments on this point were repeated with bacilli from colonies isolated from a normal Shiga culture which had been exposed to dysentery bacteriophage and then plated. The resulting, undissolved bacilli must all be resistant to some degree, but the colonies which grew were of the two distinct types noted above, and were designated sensitive resistant, or irregular, and resistant resistant, or regular. The bacilli from the regular colonies were not dissolved by the dysentery bacteriophage, nor did their broth filtrate dissolve normal Shiga bacilli of the original strain, or the bacilli of the irregular colonies. On bacilli from the regular colonies some lysis resulted. The bacilli from the irregular colonies, on the other hand, were dissolved by the dysentery phage and by their homologous filtrate, which also dissolved normal dysentery bacilli and acted to some extent on the bacilli of the regular type of colony. This was explained by the assumption that the culture of regular, resistant bacilli was not absolutely pure in the sense that some less resistant bacilli were still present. By plating, this was proved to be correct, for both regular and irregular colonies developed from a broth tube of regular bacilli which had been in contact with lytic fluid over night. The regular colonies fished from this plate were again subjected to the action of the strong phage, and plated after 4 days. The resulting colonies were all of the regular type, and were not dissolved by the strong dysentery phage or by filtrates from regular or irregular types of bacilli. It is possible then to obtain a pure, resistant type of dysentery bacilli by repeated elimination of sensitive bacilli from the strain, these resistant bacilli having been present originally.

The sensitive type of dysentery bacillus, after nine generations of broth and plate subculturing, was found to have become more resistant, as shown by the fact that the bacilli of the ninth generation were acted upon less strongly by the antidysenteric phage and by the filtrate from the first generation of irregular bacilli. The filtrate

from the ninth generation was also less lysogenic than the filtrate from the first generation had been.

The sensitive bacilli gradually die out by the action of the lytic substance they carry, and gradually resistant bacilli only are left. The tendency then is for cultures of sensitive bacilli to become more resistant and for resistant bacilli to remain resistant. This is also demonstrated by the colonies on repeated subcultures (Figs. 5 and 6).

#### *The Lytic Reaction under Anaerobic Conditions.*

The lytic action proceeds as rapidly and as completely in the absence of oxygen as in its presence. Deep broth cultures with rabbit kidney and sealed with vaseline were inoculated with dysentery bacilli and antidysenteric phage, and growth was prevented as it was in the aerobic controls.

#### *Morphology.*

The sensitive strains are composed of short bacilli and many coccoid and round, swollen forms. The resistant strains are made up of regular, equal sized, and evenly staining bacilli, with some larger forms, but few, if any, coccoid or swollen, round forms. Threads are seen in older cultures of the more resistant types.

#### *Virulence.*

Rabbits inoculated with minute doses of early broth cultures of the resistant bacilli became paralyzed within 48 hours, and did not recover. Cultures of the sensitive bacilli required five times as great a dose to produce similar results. Apparently the exotoxin of the resistant type of dysentery bacilli is much stronger than that of the sensitive type.

#### *Immunization.*

Rabbits were immunized with resistant strains of Shiga dysentery bacilli, with normal strains, and with Shiga bacteriophage. Agglutination tests with the resulting sera brought out the fact that normal, irregular or sensitive, and regular or resistant strains are agglutinated well, the resistant somewhat less so than the sensitive. But a highly resistant strain was not agglutinated even by the sera made with



ordinarily resistant strains. The highly resistant strain killed the animals before they developed immune bodies in their sera.

The animals immunized with antidysenteric phage agglutinated normal dysentery strains in dilution of 1:200, sensitive strains in 1:1,000, and resistant strains in 1:100, while the highly resistant strain was not agglutinated.

Evidence which shows that d'Hérelle's phenomenon goes on in the human intestine in a patient with dysentery was recently obtained. At autopsy on an infant 9 months old, who died of bronchopneumonia, a severe pseudomembranous enterocolitis was found. The child had only been under observation for 48 hours, but a history of intestinal symptoms was obtained. By proctoscopic examination on the day before death ulcers were seen in the rectum and a specimen of feces was obtained and plated. On the plates Flexner dysentery bacillus colonies developed with marked, irregular, indented edges. A few small, regular colonies were also noted. Fishings from both kinds of colonies were made, and a sensitive and a resistant strain was obtained. The plates made directly from the rectal specimen were very similar to those obtained in our experiments on the action of the antidysentery lytic fluid on dysentery bacilli. Whether, if a complicating pneumonia had not intervened, the child would have recovered from the dysentery infection or whether the resistant dysentery bacilli were present in sufficient numbers to prevent recovery in spite of the active lysis going on, remains a question.

#### SUMMARY.

It has been shown that a lytic fluid for dysentery bacilli can be obtained from the peritoneum of the guinea pig by intraperitoneal inoculation of live dysentery bacilli, and that the lytic action of such a fluid is not strictly specific, but that it exerts a group action on the dysentery-colon-typhoid group of bacilli. A lytic fluid with similar effects was obtained from a child dying of Flexner dysentery infection, and an anti-colon bacillus lytic fluid from a child who died of intussusception with colon bacillus peritonitis.

The action of the lytic fluid on the dysentery bacilli, both *in vivo* and *in vitro*, is to divide the culture into sensitive and resistant strains, and the latter can be carried to a degree of very marked, if not complete resistance to lysis. Such resistant strains are not lysogenic, nor are

they agglutinable. The sensitive strains are lysogenic and agglutinable. Varying degrees of sensitive and resistant bacilli exist in a single culture. The sensitive bacilli gradually lose the lysogenic property which they acquired under special conditions, but the very resistant bacilli never acquire that property. It is conceivable that the resistant strains are responsible for the untoward outcome of disease in human beings. Theoretically the administration of lytic fluid should rid the intestinal tract of most of the infecting bacilli, and only if completely resistant bacilli in large numbers remain unacted on is the outcome of the disease a fatal one.

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#### EXPLANATION OF PLATES.

##### PLATE 35.

- FIG. 1. Effect of the bacteriophage on an agar slope culture of *B. dysenteriae*.  
FIG. 2. Agar plate of a culture of *B. dysenteriae*.

##### PLATE 36.

- FIG. 3. Regular colonies of *B. dysenteriae* after the action of the bacteriophage on a normal strain.  
FIG. 4. Irregular colonies of *B. dysenteriae* after the action of the bacteriophage on a normal strain.

##### PLATE 37.

- FIG. 5. Regular colonies of *B. dysenteriae* after 40 generations of growth on agar plates.  
FIG. 6. Irregular colonies of *B. dysenteriae* after 40 generations of growth on agar plates.

##### PLATE 38.

- FIG. 7. Irregular colonies of *B. dysenteriae*; third generation; thin edges and hollowed centers.

##### PLATE 39.

- FIG. 8. (a) Regular colonies of *B. dysenteriae*; 40th generation. (b) Irregular colonies; 40th generation; irregular edge and heaped up center.



FIG. 1.



FIG. 2.

(Wollstein: Phenomenon of d'Hérelle.)





FIG. 4.

FIG. 3.

(Wollstein: Phenomenon of d'Hérelle.)





FIG. 6.

FIG. 5.

(Wollstein: Phenomenon of d'Hérelle.)







FIG. 7.

(Wollstein: Phenomenon of d'Hérelle.)



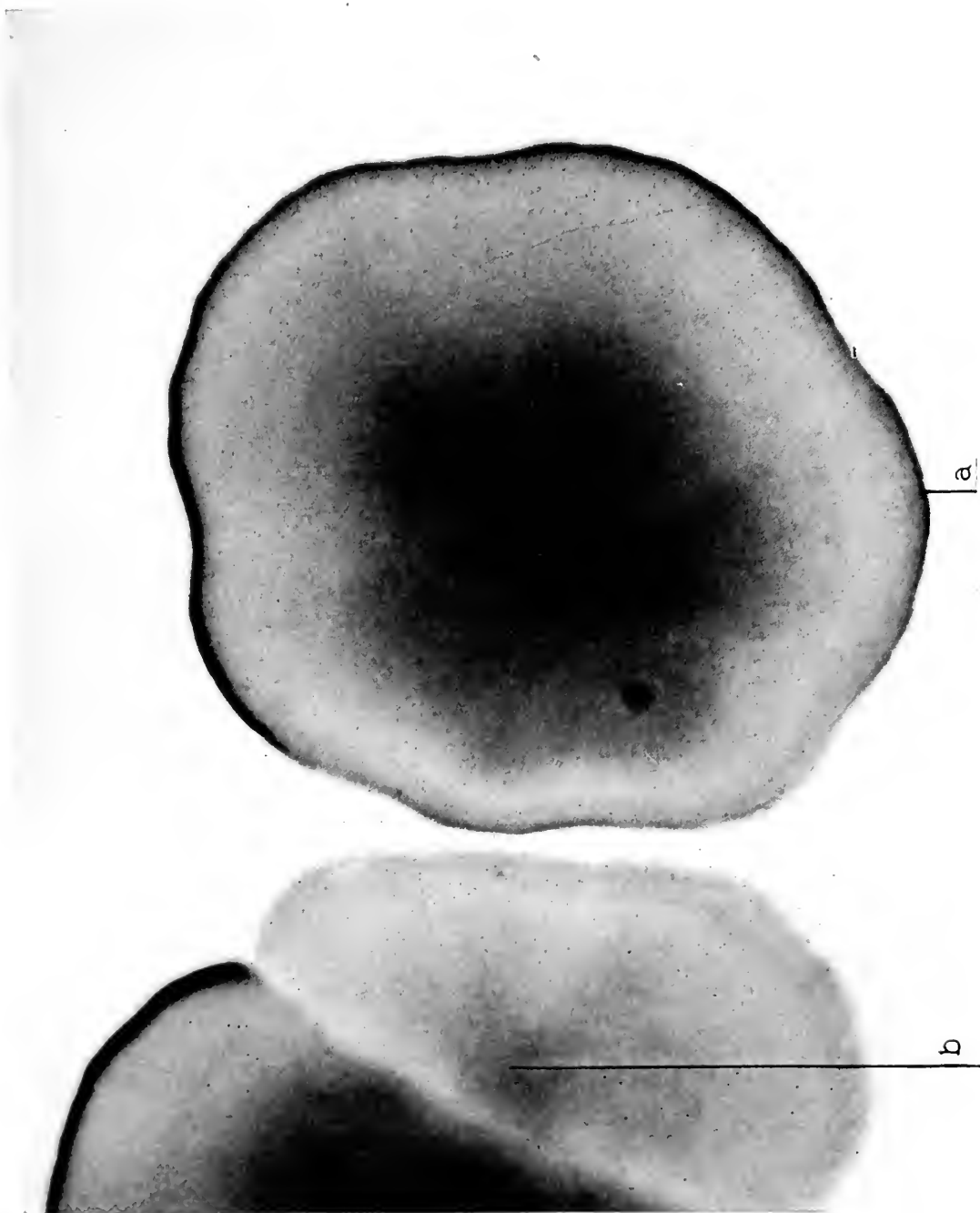


FIG. 8.

(Wollstein: Phenomenon of d'Hérelle.)



## THE BIOLOGICAL AND THE SEROLOGICAL REACTIONS OF INFLUENZA BACILLI PRODUCING MENINGITIS.

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Influenzal meningitis has been drawn into the controversy over the etiology of influenza, first by one faction and then by another, without much justification or proof by either. The relation of influenza bacilli to the disease influenza may not be settled for a long time and certainly not before much more is known about the biological and the serological behavior of these organisms. The study of influenza bacilli from the throats, sputum, and lungs of individuals with and without influenza has led to numerous opinions about the unity or multiplicity of strains of these bacilli. Even different competent workers (1) using the same strains do not obtain similar results. There must be some cause, which perhaps has not yet been discovered, to account for all these discrepancies. Since there are so many opinions about the relation of *Bacillus influenzae* to influenza and about the relation of one influenza bacillus to another, it was considered wise to select first for study a small group of the organisms which were known positively to be pathogenic. Influenza bacilli which had produced meningitis offered the group sought.

No attempt will be made at this time to cover the various ideas about influenza bacilli in general. The work of only a few investigators will be cited to show the multiplicity of opinions even about *B. influenzae* producing meningitis. Slawyk (2) isolated an influenza bacillus from the blood and spinal fluid of a child with meningitis. This organism was seen by Pfeiffer, who agreed that it was *B. influenzae*. Cohen (3) recovered from the spinal fluid and blood of patients with meningitis an organism similar to the influenza bacillus, except that it caused septicemia in rabbits, and because of this difference in virulence and evidence obtained by protection experiments, he believed the two organisms were not the same. Wollstein (4, 5) in her studies on influenzal meningitis concluded that

respiratory and meningeal strains of *B. influenzae* were more or less identical culturally and immunologically, varying only in virulence. Davis (6) found no difference in the virulence of respiratory and meningeal strains. Anderson and Schultz (7) isolated five strains of influenza bacilli from one patient with meningitis, one each from the spinal fluid, blood, nose, nasopharynx, and throat, and reported them to be serologically different. This is a rather unusual finding in view of Wollstein's (4) work, who showed that *B. influenzae* injected intravenously into rabbits and subdurally into monkeys could be recovered from the mucous membranes of the upper respiratory tract.

For a long time influenza bacilli were considered alike culturally, but lately many have shown that they can be subdivided by biological reactions. Wadsworth and Wheeler (8), Jordan (9), Stillman and Bourn (10), Rivers (11), and Rivers and Leuschner (12) have contributed to this work. The bacilli also were generally accepted as being hemoglobinophilic or hemophilic, but now there is good reason to believe that they are not hemoglobinophilic (13-16), and possibly not even hemophilic (17). At any rate the strains studied in this work meet all the old criteria of influenza bacilli—they are Gram-negative, non-motile, aerobic, pleomorphic bacilli which do not grow on ordinary nutrient or meat infusion agar when in pure culture.

Thirteen strains of *Bacillus influenzae* isolated from spinal fluids of patients who died of influenzal meningitis were used. Seven of these (Nos. 7, 8, 9, 10, 11, 12, and 16) were furnished by Dr. A. W. Williams and were from a collection made by the Department of Health of the City of New York during a period of 7 years, 1913-20. Povitzky and Denny (18) have already published work on these seven strains. The fact that the results presented here do not coincide entirely with theirs places the authors under no less of an obligation to the New York workers for supplying most of the strains studied. Strain 16 was obtained from Dr. Wollstein and may be the same as Strain 6 obtained directly from Dr. Wollstein by this laboratory. Proof, however, that these two strains came from the same patient is lacking. In this work these have been considered as separate strains. No. 17 was recently furnished by Dr. E. O. Jordan who received it from Dr. D. J. Davis, of the University of Illinois, in November, 1920. Strains 1, 2, 14, and 15 were isolated here from patients in Dr. John Howland's clinic. For comparison four blood culture strains from children with pneumonia were used. No. 3 was obtained in pure culture from a child who later recovered; Strains 4 and 5, mixed with Type IV pneumococci, and No. 13 were obtained at autopsies. A list of the strains is given in Table I.

TABLE I.  
*List of Strains.*

Strain No.	Label when received.	Source.	Date of isolation.	From whom received.
1		Meninges; influenzal meningitis.	1920	Rivers.
2		" " "	1920	"
3		Antemortem blood culture; pneumonia.	1920	"
4		Postmortem blood culture; pneumonia.	1920	"
5		Postmortem blood culture; pneumonia.	1920	"
6	B. inf. Men. 1917	Meninges; influenzal meningitis.	1917	Wollstein.
7	Z	" " "	1913-14	Williams.
8	747	" " "	1913-14	"
9	6741	" " "	1918	"
10	6746	" " "	1918	"
11	6827	" " "	1919	"
12	6745	" " "	1918	"
13		Postmortem blood culture; pneumonia.	1920	Rivers.
14		Meninges; influenzal meningitis.	1920	"
15		" " "	1919	"
16	B. I. W.	" " "	1917(?)	Williams.
17	Pfeiffer No. 26	" " "	Received by Dr. Jordan 1920.	Jordan.

*Biological Reactions.*

One particular kind of medium was used so much in the study of cultural characteristics and serological reactions that it will be described in detail and hereafter will be spoken of as hemo-peptone water for convenience.

Peptone (Fairchild's) ..... 20 gm.

Sodium chloride ..... 5 gm.

Distilled water ..... 1,000 cc.

Boiled and titrated to pH 7.4.

10 cc. of washed red blood cells added and heated to 95°C.

Filtered through paper.

Sterilized through a Mandler filter. Tubed or flaked in required amounts and incubated for sterility.

This medium is perfectly clear, contains very little protein as it will not cloud on boiling or autoclaving, and supports a splendid growth of influenza bacilli.

*Production of Indole.*—Tubes of hemo-peptone water, after inoculation and incubation for 3 to 10 days, were extracted with ether. This ether extract was decanted and tested with Ehrlich's reagent for indole. All the meningitic strains except No. 10 gave a positive test, while this was true of only two (Nos. 3 and 13) of the four blood culture strains. No. 16, which Povitzky and Denny (18) said failed to produce indole, did so about three-fourths of the time when hemo-peptone water alone was used, and all the time when tryptic digest of milk was added to the hemo-peptone water. No. 10 in the meningitic group and Nos. 4 and 5 from the blood culture group never under any conditions produced indole.

*Reduction of Nitrates.*—Hemo-peptone water plus potassium nitrate was inoculated, incubated 5 days, and then tested with the sulfanilic acid and naphthylamine reagent for nitrites. All the strains regularly reduced nitrates. In 1920, Rivers (11) stated that some influenza bacilli did and others did not reduce nitrates to nitrites. At that time he suggested that further work might show nitrite formation to be a common characteristic of these bacilli. Meat infusion broth, blood, and potassium nitrate were the medium used then. On this medium nitrate reduction takes place irregularly. If hemo-peptone water plus potassium nitrate be used, however, nitrites are formed regularly by all influenza bacilli so far tested.

*Hemolysis.*—None of these strains hemolyze red blood cells either in solid or in liquid media.

*Production of Amylase.*—A starch-splitting ferment is not produced by any of these strains.

*Fermentation of Sugars.*—For many years influenza bacilli were considered unable to ferment any of the sugars. Lately a number of American (8, 10, 18) and German (19, 20) workers have reported positive fermentations with various kinds of media and sugars. Stillman and Bourn (10) have done the best work and have reported many of the difficulties encountered in fermenting sugars with these bacilli.



In 1920, Rivers (11) stated that he could get no positive fermentations. At that time meat infusion broth plus fresh rabbit blood was used as the medium to which the test sugars were added. Since then it has been found that this is a very unsatisfactory way to test sugar fermentations, because quite often there was no evidence of the fermentation of glucose. At other times when acid was formed the reaction was more strongly marked in the control tubes to which no sugar had been added. Obviously this was an unsatisfactory method if any dependable results were to be obtained. A more suitable basic medium was sought to which the sugars could be added. The hemo-peptone water described above was used with some success, but even with this medium the results were not so regular as desired.

The following medium was then tried.

Peptone (Fairchild's) .....	20 gm.
Sodium chloride .....	5 gm.
Agar-agar .....	15 gm.
Distilled water .....	1,000 cc.

This was boiled, titrated to pH 7.4, filtered, and autoclaved in 100 cc. quantities. After it was taken from the autoclave and while it was still at 95°C., 1 cc. of washed red blood cells, 10 cc. of a 10 per cent solution of the required sugars, and enough 25 per cent alcoholic solution of brom-cresol purple to give a good color, were added. While still warm the medium was tubed and later incubated for sterility. It is desirable to make the medium so that there is a good deal of water of condensation. The coagulated blood cells, it is true, give the medium a dirty appearance but do not interfere with the results. Later a clear medium was made by adding to the peptone agar 15 to 20 cc. of an autoclaved blood clot extract and 15 to 20 cc. of a filter-sterilized yeast extract. When glucose and brom-cresol purple were added to this clear agar a better looking medium was the result and fermentation of the sugar took place. Eventually some kind of clear medium such as that just described may be used, but the findings in this paper are based on the results obtained upon the muddy looking solid medium. Readings were made daily for 10 days.

Sugar fermentations were more prompt and regular when the solid medium was used instead of the hemo-peptone water; for example, none of the strains fermented filter-sterilized maltose in a liquid medium, while Strain 4 always fermented it in the solid medium. (Autoclaved maltose gave very irregular results, probably because it is so easily hydrolyzed.) The fructose tubes were distinctly acid after 48 hours, but later often returned to about the same reaction as that of the controls. The reaction of some of the unfermentable sugar media, lactose and rhamnose for example, at times was made distinctly more alkaline by the growth of these bacilli. Apparently influenza bacilli under the proper conditions may form both acid and alkali and the final reaction is the resultant of these two factors.

Nineteen so called sugars have been tested. Sucrose, lactose, mannitol, mannose, dulcitol, dextrin, glycerol, rhamnose, inosite, sorbitol, salicin, and inulin were not fermented by any of these strains. Acid without gas was produced by all, except No. 17, from glucose, fructose, and galactose (Table II). Upon raffinose and arabinose questionable results were obtained. All except Nos. 13 and 17 formed acid without gas from xylose. Only No. 4, a blood culture strain, fermented maltose. At a glance it can be seen that the meningitic strains with the exception of No. 17 and two of the blood culture strains gave similar fermentation reactions. Strain 17 fermented none of the sugars.

*Action on Milk and Milk-Broth.*—In 1920, Rivers (11) stated that a certain group of influenza bacilli, which contained five meningitic strains, made milk-blood-broth slightly acid in 48 hours while others made it slightly alkaline. The production of acid or alkali in milk-broth mixtures by the organisms here studied varied very much when different lots of the medium were used. At times acid was formed, at others alkali. Usually all of them acted similarly upon the same medium. This variation depended most likely on the amount of glucose in the broth used, as the same variation was noted on blood-broth mixtures alone. Dochez<sup>1</sup> has found that the glucose in different lots of meat infusion broth varies considerably, as shown by quantitative determinations. When milk with heated washed red blood cells alone was used, however, all the strains fairly regularly produced a slight amount of alkali. The use of milk-blood-broth seems inad-

<sup>1</sup> Dochez, A. R., personal communication.

visible as a routine procedure. If milk is to be used at all, skimmed milk without broth should be chosen, as the results will be more constant and reliable.

TABLE II.  
*Cultural Characteristics.*

Strain No.	Source.	Indole production.	Reduction of nitrates.	Glucose.	Fructose.	Galactose.	Maltose.	Xylose.	Milk-broth.	Milk.
1	Meninges; influenzal meningitis.	+	+	+	+	+	—	+	?	Alkali.
2	“ “	+	+	+	+	+	—	+	?	“
6	“ “	+	+	+	+	+	—	+	?	“
7	“ “	+	+	+	+	+	—	+	?	“
8	“ “	+	+	+	+	+	—	+	?	“
9	“ “	+	+	+	+	+	—	+	?	“
10	“ “	—	+	+	+	+	—	+	?	“
11	“ “	+	+	+	+	+	—	+	?	“
12	“ “	+	+	+	+	+	—	+	?	“
14	“ “	+	+	+	+	+	—	+	?	“
15	“ “	+	+	+	+	+	—	+	?	“
16	“ “	+	+	+	+	+	—	+	?	“
17	“ “	+	+	—	—	—	—	—	0	0
3	Blood culture; pneumonia.	+	+	+	+	+	—	+	?	Alkali.
4	“ “ “	—	+	+	+	+	+	+	?	“
5	“ “ “	—	+	+	+	+	—	+	?	“
13	“ “ “	+	+	+	+	+	—	—	?	“

The most important positive cultural characteristics are given in this table. Under the sugars + indicates acid production without gas, — indicates production of neither acid nor gas. Under milk-broth ? indicates that the reaction sometimes became more acid, at other times more alkaline, 0 indicates that the test was not made.

### *Serological Reactions.*

For the study of serological relations the agglutination reaction was chosen, chiefly because it can be verified and extended by the absorption of agglutinin test. Moreover, in the majority of the recent work on influenza bacilli this reaction has been used and it was felt that whatever correlation obtained with meningitic strains would stand out all the more sharply from the heterogeneity of respiratory strains.

*Preparation of Immune Sera.*—For the study of agglutinations and absorptions monovalent antisera were prepared from rabbits with Strains 1, 3, 4, 5, 6, 8, 10, 11, 13, 14, and 15, and from sheep with Strain 9. Preliminary bleedings were titered for normal agglutinins. Most of the normal sera agglutinated none of the various strains in a greater dilution than 1:20. Several, however, agglutinated a few of the strains in a dilution of 1:40. In subsequent work normal agglutinins did not interfere as 1:100 was the first dilution in each series.

The technique of immunizing the rabbits consisted in intravenous inoculation of live 24 hour cultures in cooked rabbit blood meat infusion broth, beginning with 0.25 cc. and gradually increasing the dose up to 1 to 1.5 cc. Three series of injections were made on 3 successive days. Each series was followed by a rest of 4 days. 5 days after the last inoculation test bleedings were titered for agglutinins. The animals whose sera showed a titer of 1:1,200 or over were bled and the serum was collected and preserved with 0.5 per cent phenol. Many sera had a titer of 1:3,000 or over. Animals whose sera were unsatisfactory and in some cases animals surviving the bleeding were given more inoculations. The sheep was immunized by giving intravenous inoculations of live cultures of Strain 9 in hemo-peptone water made with sheep blood. The initial dose of 0.5 cc. was gradually increased to 20 cc. Injections of more than 20 cc. of the broth cultures were not well tolerated. Then the bacteria were collected by centrifuging, and the largest dose given was bacteria from 100 cc. of broth culture suspended in 10 cc. of salt solution. The injections were made daily for 7 days, followed by a rest of 5 to 7 days.

*Preparation of Antigens for Agglutination.*—A few preliminary experiments showed that the hemo-peptone water would be suitable for the preparation of agglutinating antigens and that formalinization favored the agglutinability of a suspension. The medium was prepared in large quantities; 200 to 300 cc. were placed in 500 cc. Erlenmeyer flasks, and incubated several days to insure sterility. The organisms from which it was desired to prepare antigens were cultivated through eight to ten daily transplants in tubes of this medium, in the expectation, as Dreyer and his coworkers (21) found for organisms of the enteric group, that there would be a loss of the tendency to spon-

taneous agglutination with an increased sensitivity to agglutination by immune sera. This was found to be true with influenza bacilli. After a number of transplants a diffuse growth was obtained in 12 to 18 hours. Then 1 cc. of these rapidly growing young cultures was inoculated into the flasks of the medium and incubated for 6 to 24 hours at 37°C. When the growth was considered sufficient, but in no case later than 24 hours after inoculation, the cultures were removed from the incubator and 0.1 per cent of commercial formalin was added. These flasks were stored in the refrigerator, being removed for daily shaking. After 7 to 10 days the antigens were found to be suitable for use. They were compared in density with a certain antigen arbitrarily chosen as a standard and diluted with sterile saline solution containing 0.1 per cent formalin. The resultant opacity was approximately that of the Oxford standard cultures.

Formalinized antigens prepared in this manner were used throughout the serological work. They proved very agglutinable, not prone to spontaneous agglutination, and, it was felt, comparable from day to day, though successive antigens from the same culture varied somewhat in sensitivity, and several of the strains always gave far more flocculable suspensions than others.

After standing 3 to 4 weeks, several of the cultures, especially those of non-meningitic origin, suddenly began to flocculate spontaneously. This phenomenon was looked for after holding an antigen for a month. Often, however, the antigens remained satisfactory for much longer periods.

*Agglutination Tests.*—With sera and antigens prepared as described a series of agglutination tests was performed. The incubation period for the test was invariably 6 hours at 55°C. in the water bath. Readings were made with the naked eye and artificial light 15 minutes after removing the tubes from the bath. The results of these tests are shown in Table III.

It is apparent (1) that with the exception of one or two doubtful results the four non-meningitic strains are agglutinatively distinct from the group, and from each other; (2) that Strain 10 is agglutinated by practically no serum save its own, which in turn agglutinates only Nos. 8 and 15; (3) that Strain 17 is not agglutinated to titer by any of the sera used; (4) that the majority of the other strains are

TABLE III.

*Results of Agglutination Tests.*

Strain No.	Serum No.												
	1	6	11	15	8	9	14	1	10	3	4	5	13
1	++	++	++	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++	++	++	++	++	++
7	++	++	++	++	++	++	++	++	++	++	++	++	++
11	++	++	++	++	++	++	++	++	++	++	++	++	++
12	++	++	++	++	++	++	++	++	++	++	++	++	++
16	++	++	++	++	++	++	++	++	++	++	++	++	++
15	++	++	++	++	++	++	++	++	++	++	++	++	++
8	++	++	++	++	++	++	++	++	++	++	++	++	++
9	++	++	++	++	++	++	++	++	++	++	++	++	++
14	++	++	++	++	++	++	++	++	++	++	++	++	++
10	++	++	++	++	++	++	++	++	++	++	++	++	++
17	0	+	0	0	+	0	0	0	0	++	0	0	++
3	++	++	++	++	++	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++	++	++	++	++	++	++
13	++	++	++	++	++	++	++	++	++	++	++	++	++

+++ indicates to titer or over; ++ three-quarter titer; + one-half titer; + any agglutination up to one-quarter titer; — no agglutination; 0 no test made. Note the marked cross-agglutination among meningitic strains. Nos. 3, 4, 5, and 13 are blood culture strains.

agglutinated by almost all the sera. Closer inspection, however, shows that Serum 1 picks out Nos. 2, 6, 7, 11, 12, and 16, and that Serum 14 agglutinates Nos. 7, 9, 8, 15, and 16, leaving Nos. 1, 2, 6, 11, and 12 less affected.

This marked degree of cross-agglutination emphasized the necessity of absorptions. Accordingly, these were begun at once without extensive repetition of agglutinations. It may be said at this point that the control agglutinations made with unabsorbed sera for comparison with absorption tests (Table IV) emphasized some of the differences pointed out above, so that it is probable that the agglutinative picture is not so confused as Table III would indicate. It is possible that by shortening the period of immunization of animals, as suggested by Tulloch (22), and using a less sensitive antigen, effective differences in agglutinations might be elicited even with this group of closely related strains.

*Absorption Tests.*—The organisms for absorption tests were grown in Kolle flasks on chocolate-blood meat infusion agar. Defibrinated human or cat blood was used as a common method of avoiding the blood used in the medium upon which the influenza bacilli had been cultivated for immunizing the animals, and the blood of the animals immunized, which were, of course, always identical. The flasks were seeded with 1 to 2 cc. of a young culture in hemo-peptone water. These cultures for seeding were transplanted daily as the impression was that such agglutinable cultures gave a growth which absorbed better than that obtained from seedings with old cultures transplanted weekly on blood agar. After the flasks had been incubated for 18 hours the growth was scraped off with a heavy nichrome spud and suspended in a minimal amount of salt solution. The resulting heavy emulsion contained at times small pieces of agar which were removed by filtration through a quadruple layer of cheese-cloth. Sedimentation was effected in the centrifuge for 30 minutes at 2,500 revolutions per minute in graduated tubes, after which the supernatant fluid was decanted and the volume of packed bacteria noted.

A few absorptions were made with the sera diluted to 1:50, but it was found that large doses of the organisms did not consistently remove the agglutinins for the absorbing strain. Then a 1:20 dilution of the sera was used with better results. To the packed bacterial

mass was added enough 1:20 serum to give two, three, or four parts of cells to one of undiluted serum, according to the titer of the serum used. This method of adding serum appeared more accurate than pipetting into each tube of material to be tested the small quantity of undiluted serum and then adding the requisite amount of salt solution. Of

TABLE IV.

*Results of Agglutination Tests with Serum 6 Absorbed with Strain 8.*

0.6 cc. of packed organisms to 0.4 cc. of serum; absorption in water bath for 3 hours at 55°C.; agglutination after absorption in water bath at 55°C. for 6 hours.

Strain No.	Salt solution control.	Dilutions of unabsorbed serum control.							
		1:100	1:200	1:400	1:800	1:1,200	1:1,600	1:2,000	1:2,400
1	—	+	+	+	+	+	+	+	+
2	—	+	+	+	+	+	+	+	+
6	—	+	+	+	+	+	+	+	+
8	—	+	+	+	+	+	+	+	—
9	—	+	+	+	+	+	—	—	—
11	—	+	+	+	+	+	+	+	+
14	—	+	+	+	+	—	—	—	—
15	—	+	+	+	+	+	+	+	+

Strain No.	Salt solution control.	Dilutions of absorbed serum.							
		1:100	1:200	1:400	1:800	1:1,200	1:1,600	1:2,000	
1	—	+	+	+	+	+	+	—	
2	—	+	+	+	+	+	+	—	
6	—	+	+	+	+	+	+	—	
8	—	—	—	—	—	—	—	—	
9	—	—	—	—	—	—	—	—	
11	—	+	+	+	+	+	—	—	
14	—	—	—	—	—	—	—	—	
15	—	+	+	+	—	—	—	—	

+ indicates agglutination; — no agglutination.

Strain 8 removes from Serum 6 the agglutinins for Nos. 8, 9, and 14.

course the final dilution would be slightly more than 1:20 and should be taken into consideration when the agglutinations are set up after absorption. Attempts to obtain the limits of specificity by using large doses of bacteria—seven or eight parts of bacteria to one part of serum—never gave a complete removal of heterologous agglutinins.



The elimination of the possibility of non-specific removal of agglutinins seemed certain as the proportion of bacteria to serum was never more than 4:1 and most of the time 2:1 or 3:1. Absorptions were made at 55°C. in the water bath for 3 hours, the tubes being shaken occasionally to insure good contact. Then the tubes were centrifuged for 30 minutes. The clear supernatant fluid was collected and stored on ice till the following day when agglutinations were set up, with the technique described above. The usual salt and serum controls were employed; the latter were made from serum which had been incubated, centrifuged, and let stand exactly as had the test material. The titer of a serum for the homologous strain was often reduced markedly, sometimes to one-fourth the control titer, when it was absorbed by a heterologous strain. These results were interpreted as negative in view of the complete removal of the agglutinins by a strain of the homologous group in similar dosage. The phenomenon occurred particularly with the meningitic strains which fell into different groups. This technique has been described at length because it is felt that an attempt has been made to standardize serological work upon influenza bacilli which may bear an important relation to the results obtained.

When Serum 6 was absorbed by Strain 8 it was found, as shown in Table IV, that the agglutinins for Nos. 8, 9, and 14 were removed, while those for the homologous strain and Nos. 1, 2, 11, and 15 were left. A similar result was obtained when Serum 6 was absorbed by Strain 9. These tests suggested at once a grouping of certain strains and it will be noted that the agglutinations in Table III may be interpreted to give support to this grouping with the exception of the behavior of Strain 14 toward Serum 9. A later agglutination test controlling absorptions from Serum 9 showed No. 14 to be agglutinated almost to the titer of this serum.

To prove a grouping each serum was absorbed with the strains which it agglutinated. The results are shown in Table V.

From Table V the following facts are apparent. (1) Strains 1, 2, 6, 7, 11, 12, and 16 are alike in removing from Sera 1, 6, and 11 agglutinins for the homologous strain. (2) Strains 8, 9, and 14 form a similar group. (3) Strain 15 is probably intermediate between these two groups and more closely allied to the second group than the other.

Strain 15 completely removes the agglutinins from no serum except its own for the homologous organisms, while on the other hand all of the strains of Group 2 and Nos. 2 and 11 of the other group remove completely the specific agglutinins from Serum 15. (4) Strain 17 falls in neither group nor with Strain 3. (5) When Strain 10 manifested agglutinative relations with either of the main groups these were not

TABLE V.

*Results of Absorption Tests.*

Groups.	Strain No.	Serum No.											
		1	6	11	15	8	9	14	10	3	4	5	13
1	1		+	+	—	—	—	—					
	2	+	+	+	+	—		—					
	6	+		+		—	—	—					
	7	+	+	+	—	—	—	—					
	11	+	+		+	—	—	—					
	12	+	+		—	—	—						
	16	+	+	+	—	—	—	—					—
Intermediate.	15	—	—	—		—	—	—	—				—
2	8	—	—	—	+		+	+	—				
	9	—	—	—	+	+		+					
	14				+	+	+						
	10		—										
	17		—			—				—			
	3												
	4												
	5		—		—								
	13				—								

+ indicates the complete removal of specific agglutinins from a serum by a strain; — indicates the opposite. Where a symbol is lacking no absorption was made. Nos. 1, 2, 6, 7, 11, 12, and 16 constitute Group 1; Nos. 8, 9, and 14 Group 2; No. 15 is intermediate.

confirmed by absorption tests. (6) Certain ambiguous agglutinative reactions of the four non-meningitic strains can be interpreted as group reactions.

## DISCUSSION.

Valentine and Cooper (23) found two groups of two strains each among 171 respiratory strains. One of these groups was enlarged by Povitzky and Denny (18) to include five strains by the study of 90 additional ones. On the other hand, the latter authors found four of seven meningitic strains to be identical. There is nothing to indicate that the significance of this difference in grouping between the meningitic and the respiratory strains was appreciated in relation to the disease influenzal meningitis. In fact it appears that they look upon it as a matter of chance, as they say: "In cases of influenzal meningitis, we were so fortunate as to have discovered a dominant type of *Bacillus influenzae*."

The grouping which we obtained is not in accord with that shown by Povitzky and Denny (18) with seven of these strains. They found that meningitic strains corresponding to our Nos. 7, 8, 9, and 16 were agglutinated by an antiserum to No. 9 and absorbed the agglutinins; that an antiserum to No. 10 agglutinated only the homologous organism; and that Nos. 11 and 12 were agglutinated by neither of the above sera nor did they absorb the specific agglutinins from Serum 9. We found, using many more sera than they, that of these seven strains, Nos. 7, 11, 12, and 16 were in one group, Nos. 8 and 9 in another group, and No. 10 stood alone. No explanation is offered for this difference in results.

The essential point, however, is that we have been able to confirm and extend their results, showing the existence of definite groups embracing the majority of the available strains of influenza bacilli isolated from cases of meningitis in which they occurred. This is especially marked in distinction to the apparent heterogeneity of respiratory strains as well as of the four blood culture strains which we have studied from children with pneumonia.

It is difficult without further work on more meningitic strains and without further careful comparison of these with many respiratory strains to state just what the results so far indicate. At any rate several possibilities are suggested. The most interesting one is that out of the large heterogeneous group of influenza bacilli a smaller group of organisms possessing similar cultural characteristics and

similar or closely allied serological reactions has become specialized parasites, which enables them to attack and produce in children a definite disease picture, influenzal meningitis. This is analogous to the meningitis produced by the meningococci which constitute a definite group among the Gram-negative diplococci. If the above possibility proves untrue the next most likely one is that certain groups of influenza bacilli more or less similar culturally and serologically, having risen to various levels of pathogenicity, can cause serious diseases in man, for example, septicemias, pneumonias, and meningitis, in this respect being similar to pneumococci and streptococci.

Out of the thirteen meningitic strains the results with only two (Nos. 10 and 17) interfere with the first possibility mentioned. Immediately the question is raised as to which will be called meningitic strains. The history of the patients from whom Nos. 10 and 17 were isolated is not available at this time. These strains were isolated, it is true, from patients with meningitis, but this may have been secondary to a mastoid infection or pneumonia. In that event the meningitis might be considered a complication of some other disease caused by a respiratory strain of *Bacillus influenzae*. Nor is it known whether these two strains were obtained in pure culture. It may be that they attacked the meninges aided by pneumococci or streptococci rather than by powers of their own pathogenicity. At least one child seen at the Harriet Lane Home had a meningitis following a mastoid infection. Cultures from the mastoid and the spinal fluid revealed a hemolytic streptococcus and an influenza bacillus. That strain was lost before this work was undertaken. It will require further study of patients and of autopsy material in connection with the study of the cultural and the serological reactions of the influenza bacilli isolated from them before it can be said definitely that there is a group of these organisms which has a predilection for the meninges, producing a meningitis as meningococci do and not secondarily to mastoiditis, sinusitis, or pneumonia.

If influenzal meningitis proves to be analogous to epidemic meningitis a further effort should be made to produce a curative serum in an attempt to lower a mortality of 90 to 95 per cent.

## CONCLUSIONS.

1. An attempt has been made to standardize the cultural and the serological work on influenza bacilli. As technique and knowledge improve cultural and serological characteristics may be shown to be different from those reported here. The results are constant enough for comparison as they were obtained under as nearly similar conditions as possible.

2. Of thirteen meningitic strains of *Bacillus influenzae* isolated by different workers during a period of 7 years, eleven are alike culturally and fall into two groups by absorption of agglutinin tests; seven are in Group 1, three in Group 2, with one intermediate strain. Two strains stand alone culturally and serologically.

3. Four blood culture strains from children with pneumonia differ from each other culturally and serologically. When these strains show a relation culturally to members of the meningitic group this is not confirmed by serological reactions. When more respiratory strains are studied there may be found among them some similar culturally and serologically to the meningitic group.

4. Evidence has been set forth in favor of the possibility that a certain group of influenza bacilli may have risen to a level of pathogenicity to produce a disease picture known as influenzal meningitis. If this be true an attempt should be made to obtain a potent serum for treatment.

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# STUDIES ON THE TUBERCULIN REACTION AND ON SPECIFIC HYPERSENSITIVENESS IN BACTERIAL INFECTION.

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PLATES 40 TO 43.

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For a number of years we have been interested in defining, if possible, the rôle which anaphylactic sensitization to bacterial cell constituents might play in the symptomatology and pathology of infectious diseases. We have studied this problem from a number of different points of view and some years ago published, with Parker (1), a series of observations on the sensitization of guinea pigs with typhoid bacilli in which we made use of the isolated uterus method of Dale.

It has been quite apparent to all workers in bacterial anaphylaxis that the sensitization of animals to the cell materials of bacteria is much more difficult than is analogous sensitization with sera, egg albumin, etc., and it has usually been assumed, and we believe correctly, that this was due to the relatively small amount of coagulable protein in the bacterial cell.

In thinking about the problems of bacterial hypersensitiveness since that time, it has seemed to us of fundamental importance to obtain, if possible, a clear understanding of skin reactions such as the tuberculin, typhoidin, mallein, etc., tests, which represent more or less specific forms of hypersensitiveness in infected animals and man but, at the same time, differ in a number of important and perhaps fundamental aspects from phenomena generally associated with true anaphylaxis.<sup>1</sup>

<sup>1</sup> By true anaphylaxis we mean the reaction of protein hypersensitiveness, in which it is now generally agreed that an antigen-antibody union is involved in which the predominant rôle is played by the sessile antibodies, and which can be

## I.

*The Two Different Types of Skin Reaction.*

A basic fact in regard to skin reactivity is the occurrence, in man and in guinea pigs, of two fundamentally different types of such reactions, both of them to a considerable degree specific, yet differing both in the nature of the completed reactions and in the time factors concerned in their development. We specify guinea pigs and man because, in rabbits, the distinction is not so clear, a matter to which further reference will be made below. The two forms to which we refer are as follows:

1. The intradermal reaction, which appears in from 2 to 3 to 15 minutes after injection of the antigen. It expresses itself in the development of a growing wheal, often surrounded by a red areola of variable size. This reaction may last from  $\frac{1}{2}$  hour to 1 or 2 hours, and fades again, usually without leaving any profound injury of the tissues. This is the reaction obtained with such substances as horse serum and has been extensively used in man to determine whether or not the particular individual was sensitive to horse serum when therapeutic injections were intended.

2. The other type of skin reaction is one in which there is no immediate effect, but in which within 4, 5, or more hours, a swelling becomes apparent which in the course of 12 to 24 hours results in a swollen, edematous area of varying intensity, often with a central necrotic spot and, occasionally, hemorrhage. This reaction may not reach its highest development until about 48 hours after the injection, and is accompanied by distinct signs of inflammation and some cell death.

passively and specifically transferred to normal animals with the serum of other animals as long as this serum contains antibodies. We do not wish to take up space with this definition, but refer the reader to the recent and excellent summaries of Doerr (Doerr, R., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, ii, 947), Coca (Coca, A. F., in Tice, F., *Practice of medicine*, New York, 1920, i, 107), and Wells (Wells, H. G., *Physiol. Rev.*, 1921, i, 44). Although differing somewhat in nomenclature and in classification, the three summaries lay down fundamental principles which are essentially alike and with which we agree.



The classical examples of such skin reactions are the tuberculin intradermal test, the typhoidin reaction, the mallein reaction, and the reactions studied by Fleischner, Meyer, and Shaw (2) with *Bacillus abortus* and *Bacillus melitensis* in guinea pigs.

As we shall see, the two reactions may occur in the same animal, after one and the same injection, but, in guinea pigs, can usually be differentiated, since one begins to fade before the other appears.

It seemed that our first task, then, was to determine whether either one of these varieties of skin reactions or perhaps both of them could be correlated with true anaphylaxis.

*Relationship of the "Immediate" Skin Reaction to Anaphylaxis.*

The immediate skin reaction has been used for some years as an index of safety before the injection of therapeutic horse serum in the specific treatment of pneumonia and other diseases. Its application in this connection was based primarily upon the publications of Moss (3), of Knox, Moss, and Brown (4), of Longcope and Rackemann (5), of Mackenzie and Leake (6), and of others. It should be noted, however, that the work of Moss and his collaborators, on which the assumption that the skin reaction was a reliable index of general hypersensitiveness is based, was done with rabbits, and in rabbits the skin manifestations of anaphylaxis, both intracutaneous and subcutaneous (Arthus phenomenon) are always in the form of delayed reactions, for reasons that we believe depend upon peculiarities of the rabbit, rather than upon fundamental differences in the nature of the reaction. This peculiarity in rabbits, however, is being investigated in our laboratory at the present time in a study on the Arthus phenomenon and will be reported upon subsequently. Meanwhile, it seemed to us necessary, since we were working with guinea pigs, to determine whether in these animals an immediate skin reaction to a protein antigen such as horse serum, egg albumin, etc., could be elicited at all, and, if so, whether its development was parallel to that of general protein hypersensitiveness.

In consequence, we carried out a number of experiments in this direction.<sup>2</sup>

<sup>2</sup> These experiments were done with the assistance of Dr. S. T. Woo and Mrs. J. T. Parker, who was carrying out skin reactions in guinea pigs for another purpose at the time.

Series of guinea pigs were given from 1 to 2 cc. of horse serum intraperitoneally. At the end of from 2 to 3 weeks, they were given intracutaneous injections of horse serum in dilutions ranging from 1 : 5 to 1 : 20. Normal controls were always injected at the same time and intravenous injections of varying quantities of horse serum were made on guinea pigs similarly sensitized in order to determine general anaphylaxis. We omit protocols since the experiments were extremely simple and we do not wish needlessly to prolong this paper.

Our results may be stated briefly as follows: Guinea pigs in the sensitized condition, that is 2 to 3 weeks after the intraperitoneal injection of 1 cc. of horse serum, will generally, though not always, show an immediate reaction to the intracutaneous injection of 0.1 cc. of a 1:10 or 1:20 dilution of horse serum.

There is a considerable increase in the size of the wheal formed at the point of injection, the increase beginning usually within 5 or 10 minutes, and increasing up to from  $\frac{1}{2}$  to 2 hours, then gradually fading. It was usually completely gone in from 2 to 4 hours and in most cases was accompanied by a red areola quite comparable to the reaction observed in human beings under similar conditions. These reactions never caused any serious or lasting injury to the tissues.

This reaction was roughly parallel to the sensitiveness of the guinea pigs to intravenous horse serum injections.

It can be produced in guinea pigs by passive sensitization, appearing on the 2nd day after the injection of the anti-horse rabbit serum almost as well as in the actively sensitized guinea pigs.

Guinea pigs that have been shocked and become antianaphylactic are desensitized to skin reactions made on the following day.

#### *Relationship of the Delayed Tuberculin Type of Skin Reaction to Anaphylaxis.*

Having thus determined that immediate skin reactions to horse serum in guinea pigs might be regarded, with reasonable certainty, as one of the manifestations of general hypersensitiveness, we next proceeded to investigate the same point in connection with the second type of reaction; namely, that represented by tuberculin, mallein reactions, etc. We selected tuberculosis and the tuberculin reaction as the first type to be studied because with tubercle bacilli it is relatively easy to produce, at will, infections of varying intensities and different speeds of progression, and since the volume of clinical

data and the careful studies of tuberculin skin reactions in guinea pigs by Baldwin, Krause, and others had cleared the way for a free approach to the basic problems. The reaction, moreover, is essentially the same type of occurrence as that following the injection of typhoidin, mallein, or abortin preparations in animals respectively infected with the organisms from which the antigens have been produced.

It is quite well known that in an interpretation of reactions such as the tuberculin reaction, the field is fairly divided between those that hold these phenomena to be manifestations of true anaphylaxis and those who deny this.

Calmette (7), who separates the tuberculin reactions distinctly from anaphylaxis, nevertheless states that tuberculous animals or man develop a lytic principle, presumably in the nature of a bacteriolysin, which goes into reaction with the specific antigen in the tuberculin preparations. This fundamentally is, in a sense, an anaphylactic conception. Friedberger has frankly regarded the phenomenon as an anaphylactic one, developing his ideas along his theories of anaphylatoxin formation.

Study of the reactions from a theoretical point of view was begun by Römer (8) in 1909, and by Baldwin (9) in 1910. Baldwin's work is fundamental, in showing that, in spite of previous assertions, guinea pigs could not be rendered skin-sensitive by implantation of porous filter capsules or celloidin capsules containing tuberculoprotein, or living tubercle bacilli. He showed that skin sensitiveness could never be produced without actual infection with living organisms. Animals treated with tuberculoprotein, however, often showed reactions to intravenous inoculation of the homologous preparation which could be recognized as anaphylactic. His conclusions can be summarized as follows:

Tuberculous animals become sensitive to anaphylactic test, but not uniformly so. There is no absolute relation between the degree of sensitiveness and the stage of the disease. Injections of the tuberculoprotein may sensitize normal guinea pigs. Sensitized guinea pigs, however, do not react to the ordinary tuberculin test, though some respond slightly to the intradermal test. He adds: "This difference between anaphylactic sensitization and tuberculin reactivity need not be fundamental, as it is possibly due to another factor as yet undetermined." His experiments on the transfer of passive anaphylaxis to tuberculoprotein were inconclusive, but it has been shown since then, by Austrian (10) and others, that passive sensitization can be attained. From a theoretical point of view the most important observation of Baldwin is the fact that there seemed to be a discrepancy between skin sensitiveness and general anaphylaxis. Krause (11), following out the work of Baldwin, confirmed and extended the above observations and established an interesting relationship between skin sensitiveness and the progress

of infection. He asserts that skin sensitiveness develops simultaneously with the development of the initial focus, increases progressively with the lesions, varies directly with the extent and intensity of the infection, and diminishes with healing. It is blunted by a general tuberculin reaction which suggests analogy to saturation, such as that which occurs in connection with anaphylaxis.

In dealing theoretically with the tuberculin reaction, especially as regards the possibility of its being an anaphylactic phenomenon, it is of great importance to determine whether or not the reaction can be transmitted passively. The evidence on this is contradictory. Only a few writers have reported positively in this connection. In 1909, Bail (12) injected finely divided tissue mash of tuberculous organs of guinea pigs into normal guinea pigs, and 24 hours later gave the animals so treated 0.5 cc. of old tuberculin, or a preparation which in this quantity had practically no effect upon normal animals. The animals prepared with the tuberculous tissue died in some cases, while controls treated with normal tissue suspensions showed no symptoms.

Helmholz (13) in the same year reported positive skin reactions in normal guinea pigs 2 to 6 days after he had injected them intraperitoneally with the defibrinated blood of tuberculous guinea pigs. Both of these observations would be of fundamental importance if they could be confirmed.

In considering the mechanism of the tuberculin reaction, it will be well to examine also the work that has been done on the typhoidin reaction. Gay and Claypole (14) believed that positive skin reactions in rabbits were parallel with the degree of immunity of the animal. They succeeded in transferring the susceptibility to typhoidin from an immune to a normal animal by inoculation of 20 cc. of typhoid-immune serum, testing 24 hours later. These experiments were repeated and confirmed by Meyer and Christiansen (15); and in their first work with rabbits, these last observers, using what they called a typhoid autolysate (by which they mean an alcohol precipitate of a heated distilled water suspension of a 48 hour agar culture), concluded that "the typhoidin and similar reactions in rabbits are anaphylactic in nature and the result of an interaction of antigen and antibody." They stated that "the logical assumption from these facts is that cutaneous hypersensitiveness is the result of bacterial protein sensitization." Later Meyer (16) found that injected rabbits react with typhoidin more intensively than do immunized rabbits, and drew the conclusion that cutaneous hypersensitiveness does not indicate that the rabbit is particularly immune, and that no definite relationship existed between agglutinins and complement-fixing antibodies and skin sensitiveness. From these first two papers of Meyer's we gather that he believed that in rabbits skin sensitiveness to typhoidin is a sign of infection, rather than of immunity, but that as stated in his own words "cutaneous hypersensitiveness of rabbits . . . is, in all probability, the result of sensitization with typho- or similar bacterial proteins." Nichols (17) also considered the typhoidin reaction as he observed it in human beings as a protein sensitization.

The apparent discrepancies between the results of Baldwin with the tuberculin reaction and those of the workers just mentioned with the analogous typhoidin reaction, probably depend upon the fact that Baldwin used guinea pigs and the other observers used rabbits. When, subsequently, Fleischner, Meyer, and Shaw (2) studied cutaneous hypersensitiveness in guinea pigs treated with repeated intraperitoneal injections of *B. abortus*, *B. typhosus*, and old tuberculin, and carried out parallel experiments with animals infected with living organisms, the conclusions that they reached coincided with those of Baldwin in the case of the tuberculin reaction. They found, in other words, that guinea pigs treated with dead bacterial proteins might become anaphylactic, but did not give skin reactions.

It will be seen from this brief review of the literature that, in spite of a great deal of careful work by experienced observers, there is still a considerable degree of confusion. We considered it best, therefore, in studying the tuberculin reaction to begin at the bottom and re-examine the conditions in all their details, substituting reactions with the isolated guinea pig uterus for the intravenous test for anaphylaxis. This point is particularly important since the intravenous injection of bacterial extracts into guinea pigs is apt to give rise to confusing symptoms; and in carrying out such experiments we have again and again felt uncertain as to whether or not an individual, mild reaction should be interpreted as a feeble manifestation of anaphylaxis, as due to toxicity of the bacterial preparation, or perhaps even as the result of the injection of finely divided colloidal particles.

The first stages of our work, then, concern themselves with a study of tuberculous guinea pigs and guinea pigs treated with various tubercle bacillus extracts, determining in both cases the relationship of intracutaneous tuberculin reactions with anaphylaxis as manifested by tests with the isolated uterus. We summarize the final procedures in order to avoid a needless account of much preliminary groping for a suitable technique.

*Technique.*—The tubercle bacillus used throughout was a human type of moderate virulence originally isolated, we believe, at Saranac. Many different methods of extraction were used, but the preparation with which most of the work was done was made as follows:

About 100 mg. of ground and powdered tubercle bacilli were shaken up in a shaking machine for 3 to 4 hours, with 200 cc. of salt solution to which had been added enough normal sodium hydroxide to give a final concentration of 0.2 per cent of the alkali. When the powder was added to this, the buffers reduced the alkalinity, which finally ranged somewhere about pH 8. After shaking, the bottles

were preserved in the ice chest, and sometimes reshaken on subsequent days. The gross particles were removed before this was used, either by filtration through a Berkefeld filter, or by high speed centrifugation, and the slightly opalescent fluid was the basic preparation finally employed in most of the experiments, and fractionated subsequently for special purposes. In sensitizing guinea pigs it was very often the unfiltered extract which was intraperitoneally injected without centrifugation.

The following method was found to be the most suitable for the sensitization of normal guinea pigs with the tubercle bacillus extracts. Young female guinea pigs of about 150 to 200 gm. weight received from 1 to 2 cc. intraperitoneally, at first on alternate but in later experiments on successive days, until ten to twelve injections had been given. It was found best to test these guinea pigs not earlier than 20 days and usually not later than 28 days after the last injection.

Skin reactions were invariably done intracutaneously with a No. 26 gauge needle on a tuberculin syringe, and it was attempted to inject about 0.05 cc., but it was found that the concentration rather than the actual amount was the thing that seemed to count most. We gauged the skin reactions largely on the size of the wheal produced, attempting to produce the same sized wheal in comparative tests. It is next to impossible in guinea pigs to inject absolutely accurate quantities in separate tests, but, as a matter of fact, the variations in quantity injected could never have been very great or significant.

The uterine tests were all done in the same way, in a bath of 200 cc. of Ringer's solution with 0.5 to 1 per cent glucose and oxygen bubbling through the bath.

### *Studies with Tuberculous Guinea Pigs.*

Guinea pigs were intraperitoneally injected with relatively large doses of tubercle bacilli, without any attempt to equalize the doses or to grade them, since we wanted to perform the test on guinea pigs with varying degrees of tuberculous involvement and to check up by autopsy after the results had been obtained.

Skin reactions were done on these animals with the undiluted but filtered extracts described above, and with old tuberculin in dilutions usually of 1 : 5 or 1 : 10, at varying intervals from the 1st day after infection. When definite skin reactions appeared, some of the guinea pigs were killed and the uteri put into the Dale apparatus, the extract mentioned above being used in most of these experiments, since, in working with old tuberculin, the margin between the non-specific action on the normal uterus and the specific action on the sensitized uterus is a relatively narrow one, a thing also found by Weil (18), who is, as far as we know, the only one who has applied this method to tuberculosis, his report dealing with some isolated experiments carried out in 1917.<sup>3</sup> A great many animals

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<sup>3</sup> Weil obtained uterine reactions with old tuberculin 9 weeks after injection with 0.001 cc. of old tuberculin, while the normal uterus did not react to less than 0.3 cc. The capacity of the bath is not given. He states that the reaction is an irregular one and occurs "at some stage of the disease."

were tested in this way, and the results were perfectly consistent, so that it is unnecessary to go into detailed study of the protocols. Whenever uterine tests were done, the tubercle bacillus extract used was titrated on the same day against normal uteri to make sure that we did not approach the non-specific limit, which varied considerably in these extracts. Also, no skin reactions were ever carried out without being accompanied by tests, with the same material in the same quantities, on normal guinea pigs, since, in working with alcohol precipitates and other chemically manipulated substances, mild non-specific reactions will occasionally appear.

The question to be answered was the following. In the course of a tuberculous infection does true anaphylaxis to tubercle bacillus products develop, and, if so, is it parallel in time and in intensity with the intradermal tuberculin reaction?

Figs. 1 to 3 illustrate the results obtained. Positive skin reactions were usually obtained within 9 or 10 days after inoculation, the earliest, observed by us, being between the 6th and 7th days. At this time the animals were never anaphylactic. Fig. 1 shows complete lack of anaphylactic reaction in the uterus in an animal 10 days after inoculation, the day after a positive skin test had been obtained. Fig. 2 shows two uteri simultaneously put into the bath, both of them 9 days after inoculation with tubercle bacilli, one giving an entirely negative skin reaction, the lower one giving a very marked +++ reaction. Neither showed a sensitive uterus.

Fig. 3 shows a different state of affairs; namely, an animal inoculated subcutaneously with tubercle bacilli and tested 3 weeks after inoculation. This animal showed a powerful anaphylactic, as well as skin reaction. These records and a considerable number of similar ones indicate: (1) that guinea pigs suffering from infection with tubercle bacilli may become both skin-reactive and anaphylactic; (2) that the skin reaction develops early and may exist without any detectable signs of general anaphylaxis as evidenced by the uterine reaction; (3) that within 3 weeks or later, after the animals are still in fairly good condition, the skin reaction and the uterine reaction may co-exist.

It is during this period that a simulated parallelism may have led others, working with a less delicate anaphylactic method, into confusion. Towards the end when the guinea pigs are moribund, both the skin reaction and general anaphylaxis may fade, another feature



which simulates parallelism; but many reactions like those above have convinced us that there is no question about the correctness of Baldwin's original contention, that the two conditions, skin reactivity (and probably, therefore, the other forms of tuberculin hypersensitivity) and anaphylaxis do not necessarily coincide.

*Studies on Guinea Pigs Sensitized with Materials from Killed Bacteria.*

The next question to be answered was that concerning the conditions existing when guinea pigs were not infected, but when they were treated with products of dead tubercle bacilli. Young female guinea pigs were prepared by the method of sensitization outlined above. They were run through in sets of six or more. Between July, 1920, and January, 1921, five sets of such guinea pigs were run through, all of them treated with ten or more injections of extract. Skin reactions were done on these animals, both during the process of sensitization, that is in the course of the 10 to 20 days during which they were being treated, and at varying times thereafter, up to the period of 3 weeks after the last injection when they were tested for uterine sensitivity.

Practically none of these guinea pigs showed typical delayed skin reactions comparable to the true tuberculin reaction. In only two cases did we see reactions which might have been regarded as moderate tuberculin reactions. It may be noted that Baldwin also observed a few exceptions to his other negative results. An example of one set in which these exceptions occurred may be worth recording since it will typify our general procedure.

*Experiments on the Sensitization of Guinea Pigs with Tuberculo-protein.*—Six guinea pigs (Nos. 1 to 6), were injected intraperitoneally with 1 cc. of unfiltered tubercle bacillus extract as follows: September 1, 3, 7, 9, 11, 13, 20, 28, 1920. The first skin reactions were done on four of the guinea pigs with two preparations of old tuberculin diluted 1 : 5, and undiluted extract similar to that injected on September 28. Negative results.

October 2. All the guinea pigs were reinjected with 2 cc. of the extract; skin reactions on this day were negative.

October 7. Skin reactions done on three of the guinea pigs at the same time with four normal controls and one tuberculous control. When these reactions



were read on October 8, Guinea Pig 2 showed what ordinarily we should have called a +++ reaction. It should be mentioned that this guinea pig was very much emaciated and we were suspicious of its having been spontaneously infected with tuberculosis. It was killed and autopsied, and a few small yellowish spots on the surface of the liver about 2 mm. in size at the base were taken for section and examined,<sup>4</sup> but could not be identified as tuberculous (we may mention that this occasional appearance of small knob-like lesions on the liver of extract-treated guinea pigs has not been uncommon). The uterus of this animal was found to be moderately sensitive. Subsequent tests of the rest of these guinea pigs showed only one other suspicious skin reaction, less marked than that on No. 2.

The isolated uteri of these extract-treated guinea pigs were, with very few exceptions, found to be sensitive to extract anywhere from 20 to 30 days after the last injection. Fig. 4 shows two guinea pigs in which positive, typical reactions were obtained with the uteri with 4 cc. of a rather weak extract which in these quantities had no effect upon normal uteri. In both of these animals, the skin reactions had been consistently negative.

It may be noted in passing, however, that although these extract-treated guinea pigs did not show the typical tuberculin reaction with well defined swollen areolæ, etc., after 24 hours, they did occasionally show an immediate reaction not incomparable to the immediate reaction described above for horse serum. Whenever such reactions were observed, they occurred at about the time when the guinea pigs showed anaphylactic reaction to uterine test. This is mentioned in passing as showing that even with the products of the tubercle bacillus the two kinds of skin reaction can be elicited.

Moreover, the two graphic records shown in Fig. 4 are only two of very many in which typical uterine reactions were elicited in guinea pigs similarly prepared with considerably smaller quantities of various kinds of tubercle bacillus preparations. It will be seen in the lower record that a repetition of the extract showed the uterus to be desensitized, a matter which has been noted many times.

Therefore, from a considerable number of experiments similar to those described in perfect accord with the results obtained by Baldwin by other methods, we may conclude that delayed reactions, of the tuberculin reaction type, may develop independently of generalized anaphylaxis in the ordinary sense in which this word is used, may be

<sup>4</sup> The examination was made by Dr. Frederick Parker.

present in tuberculous guinea pigs before anaphylaxis to tuberculo-protein has developed, and are with very few exceptions entirely lacking in guinea pigs rendered anaphylactic by sensitization with tubercle bacillus products.

These facts, then, definitely confirm the opinion, first clearly advanced by Baldwin, that tuberculin hypersensitiveness may develop independently of general tuberculo-protein anaphylaxis, and that the former type of hypersensitiveness is associated particularly, perhaps solely, with the existence of an infection. This, too, is in complete keeping with the experience of Fleischner, Meyer, and Shaw who found that intradermal tests were positive only in guinea pigs infected with the bacillus of bovine abortion, but consistently negative in these animals intensively immunized by intraperitoneal injections of dead organisms, or with extracts of organisms.

## II.

The fundamental facts may then be stated as follows: Under the influence of contact with living tubercle bacilli, and probably other bacteria, two distinct varieties of hypersensitiveness develop in guinea pigs. One of them, true anaphylactic hypersensitiveness, develops late and can be readily induced by appropriate treatment with dead bacterial materials, extracts, etc. The other, the typical tuberculin reaction (and probably the mallein, typhoidin, and abortin reactions) may be regarded as infection phenomena, for, while we may eventually succeed in inducing these reactions by modified methods of sensitization,<sup>5</sup> they are most easily and characteristically elicited as soon as an infection is established, while, to date, they have but rarely and atypically followed upon any form of artificial sensitization. And yet, in spite of the apparent mutual independence of these two forms of hypersensitiveness, both reactions, or either one of them alone, may be elicited with one and the same bacterial extract in appropriately prepared animals.

This forces us to inquire whether a single constituent of such bacterial extracts is responsible for both types of reactions, or whether it is possible to separate, from such preparations, two functionally

<sup>5</sup> See below.

distinct fractions, one capable of eliciting the typical anaphylactic response, the other active in the tuberculin sense.

The latter opinion is rendered likely by the generally accepted view that the true anaphylactinogens are proteins, whereas most of the work done with tuberculin seems to indicate that these substances are of a simpler structure.

Löwenstein and Pick (19) among others conclude that the active substance in tuberculin is a protein-free, alcohol-precipitable substance which is not coagulated by boiling either in neutral or acid reaction, cannot be precipitated with ammonium sulfate, gives a negative biuret reaction, but is precipitated with tannic acid. We will not go into the details of analysis, but their final conclusion is that the active substance is not a protein, but belongs in the class of protein split products which Fischer has spoken of as "polypeptides." They claim that dialysis of the concentrated tuberculin solution weakens it after a short time, and that after prolonged dialysis its activity is entirely lost; they do not, however, state the nature of the membranes used, nor the particular conditions under which dialysis was performed. The active tuberculin substance, moreover, which Calmette uses for his ophthalmoreaction is the white, flocculent, and highly soluble substance which is precipitated out of tuberculin solutions upon the addition of ten or twenty volumes of absolute alcohol.

Along the lines of these investigations we proceeded to attempt separation of the various substances which might be obtained from tubercle bacillus extracts.

#### *Attempts at Chemical Separation of the Tuberculin from the Anaphylactinogen.*

From powdered tubercle bacilli of the human type<sup>6</sup> we produced extracts by shaking in a 0.02 per cent sodium hydroxide solution in physiological salt solution. 3 or 4 hours shaking and perhaps a day or so in the ice chest sufficed to bring a considerable amount of the material into solution. This extract was centrifugalized until all the particles had been removed and a moderately opalescent supernatant fluid was decanted.

This material, upon being acidified to approximately pH 5 to 6, with 2 per cent acetic acid in the cold, became turbid and soon precipitated in large flakes. Further acidification up to pH 4 did not redissolve these flakes. The precipitate represented the bulk of the dissolved substance in the extracts. The precipitate could be redissolved in a slightly alkaline salt solution and reprecipitated with

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<sup>6</sup> These bacilli were kindly furnished by Dr. S. A. Petroff of Saranac.

acid. Because of their precipitability by acetic acid in the cold, we designated these substances as nucleoproteins or phosphoproteins, although we do not wish to commit ourselves, chemically, since we are aware of the indefiniteness of these biochemical terms and realize fully our incompetence to deal authoritatively with a chemical problem of such difficulty, without further intensive study.

After removal of these acid-precipitable substances by centrifugation and filtration through Berkefeld candles, the fluid was brought to a boil in the acid condition, and sometimes a very faint turbidity developed which was taken to represent the presence of coagulable protein, albumin or globulin, or both. These precipitates were so fine and slight that only Berkefeld filtration would remove them, and even this was not always completely successful.

The fluid was then neutralized; that is, brought to an approximate pH of 7.

When this neutral fluid was filtered hot, we observed on numerous occasions that a slight precipitate developed over night in the ice chest, and that this precipitate redissolved on heating, a point which indicated the possibility that the tubercle bacilli may contain Bence-Jones protein. This point, however, will need further chemical analysis, a task which we have not yet had time to undertake. The water-clear fluid which was left after removal of all these substances gave in all cases a very definite precipitate with alcohol. The precipitate could be thrown down, collected, and redissolved in water with salt solution, and like the similar precipitate obtained from crude tuberculin was astonishingly soluble.

This final water-clear material gave no biuret reaction, and usually gave no sulfosalicylic acid reaction, though occasionally this was very faint; in most cases it gave no Millon reaction, was not clouded on boiling with acid, and usually gave no xanthoprotein reaction, though in some cases a slightly yellowish color appeared when the ammonia was added in the second part of the test. This material, for want of a better name, we speak of as the "proteose" residue.

Having thus fractionated the original tubercle bacillus extract, we proceeded to carry out biological reactions with various parts, comparing chiefly the whole extracts with the dissolved substances which precipitated with acid in the cold (nucleoproteins) and with the final solutions from which the acid-precipitable, acid- and heat-coagulable substances and the material suspected of being Bence-Jones protein had been removed (proteose residue).

We have done a large number of skin reactions on tuberculous guinea pigs with the apparently protein-free proteose residue, always controlling with tests on normal guinea pigs. This precaution is absolutely necessary, since we have found (as we were warned to expect by Baldwin) that repeated alcohol precipitation or prolonged boiling in an excessively acid reaction (pH 4 or below) may eventually

lead to non-specific toxicity, both for the skin test and for the isolated uterus.

Invariably this final proteose material gave skin reactions, often quite as powerful, and never more than slightly less than the original whole material. And, indeed, it was to be expected that a certain amount of the tuberculin active substance might be carried down in the flocculent precipitate formed when the acid is first added, so that the solution is thus deprived of a certain percentage of its activity. The protocol presented in Table I is an example of experiments of this kind.

TABLE I.  
*Skin Reactions Read after 24 Hours.*

No. of tuberculous guinea pigs.	Whole extract 1:2.	Proteose 1:2.	Whole extract 1:5.	Proteose 1:5.
7	++++	++++	+++	++
8	+++	++	+++	+++
9	+++	++	+++	++
	Whole extract undiluted.	Proteose undiluted.		
10	++++	+++		
11	+++	+++		

Two normal controls show no non-specific reactions.

++++ indicates a very marked reaction; +++ a large reaction, slightly less than the preceding; ++ a moderate reaction; + a definite but faint reaction; ± doubtful; — negative.

Reactions of this nature, often repeated, indicate definitely that in removing the acid-precipitable and the coagulable protein substances from the tubercle bacillus extracts, we did not remove any considerable part of the skin-reactive substance. Moreover, they justify the assumption that, in the tuberculin skin reactions, the antigen is not necessarily a protein substance, but probably belongs in the category of the so called proteoses or perhaps polypeptides.

It must be stated, however, that the nucleoprotein fraction, precipitated from the original extracts with acid in the cold, always retained tuberculin activity. In spite of repeated reprecipitation and resolution, we have never succeeded in entirely removing the capacity

of inducing skin reactions of the tuberculin type from these acid-precipitable substances. This may be due to the adsorption of the proteose material by the heavy flakes of the precipitate. On the other hand, subsequent experiments suggested the possibility that these nucleoproteins (?) which constitute the bulk of the soluble material of the bacterial cell might represent the mother substance from which the proteose materials are derived.

However this may be, the proteoses alone are fully capable of giving rise to the typical tuberculin reaction in the infected guinea pigs.

This being the case, what is the effect of this proteose fraction upon the isolated uterus of the extract-sensitized or anaphylactic guinea pig in which no tuberculin skin reaction can be elicited?

In these experiments our results were often not so clean-cut as they might have been. Nevertheless, in a considerable number of tests there was indication that the proteose fraction, powerfully skin-reactive, had little or no effect upon the uteri of extract-sensitized animals, which contracted powerfully when brought in contact with the whole extracts or the acid-precipitable fractions in amounts that had no effect upon the normal uterus.

Fig. 5 shows the uterus of a guinea pig, sensitized with extract, which was unaffected by 2 and 2.5 cc. of the proteose fraction, but contracted powerfully and went into a spasm when 1.5 cc. of the whole extract were added to the bath.

Fig. 6 shows a similar experiment in which 2.5 cc. of the proteose fraction had no effect, whereas as little as 1 cc. of the acid-precipitable fraction produced a strong contraction and spasm.

A number of records (six) as sharp and unambiguous as the above were obtained. But in addition, we must state that on three occasions the proteose material contracted the uteri of highly sensitized and of tuberculous guinea pigs when relatively large amounts of proteose were used. Whether this means that we had not freed the proteose completely of protein, or whether it signifies that, with sufficiently energetic treatment, the proteoses, too, may form antibodies, is a question that cannot be definitely answered. We incline to the latter view for reasons that will be discussed below.

*Notes on the Dialysis of Tuberculin.*

It will be remembered that the chief clinical difference between the immediate skin reaction of the anaphylactic type and the tuberculin type of reaction is the fact that the former consists in a rapidly developing urticaria-like swelling which disappears in a relatively short time, and leaves little or no injury behind. The latter, however, comes on slowly, and when once developed is accompanied by manifestations of inflammation and, eventually, profound injury of cells, often with cell death, a state of affairs which at least suggests the penetration of the injurious substances into the cells themselves. This, taken together with our knowledge of the relatively simpler structure of the tuberculin substances and Löwenstein and Pick's claims concerning their diffusibility, suggests a number of interesting lines of reasoning.

Is it not possible that the diffusibility of antigenic substances is a very important factor not only in the reaction of these diffusible substances with the cells, but also in the formation of antibodies? Is it not likely that substances which are practically non-diffusible should be excluded from direct reaction with the tissue cell and that the mechanism of antibody formation is a device for a reaction with non-diffusible materials? And, if this is so, is it reasonable to suppose that the reaction of the body to substances which are relatively more diffusible should become increasingly intracellular, as diffusibility increases, and that antibody formation, in the usual sense of the word, should, therefore, become less and less essential as relative diffusibility increases? Such a view would explain at the same time the apparent intracellular nature of such reactions as the tuberculin reaction and the difficulties encountered in attempts at passive transfer of such sensitiveness. Accordingly, we thought it worth while to attempt to separate by dialysis the substance which produced the tuberculin skin reaction from that which caused typical anaphylaxis.

In planning experiments upon the diffusion of antigenic substances through semipermeable membranes, we are quite aware of the many pitfalls which entrance into such a field on the part of the biologist implies. We know that it is by no means proven that the living cell membrane is at all permeable to substances like the proteoses and that experiments with dialysis *in vitro* may lack some of the essential criteria that govern dialysis in the animal body. Nevertheless, it seemed worth while to investigate the diffusibility of the tuberculin active substances. We at first tried to work with membranes graded by the methods of Brown (20) and of Gates (21). But since the differences in diffusibility of proteins and the proteose-like substances with which we are concerned can be very slight ones only, we abandoned this and worked empirically, attempting, in many tests, to find membranes which would let through the tuberculin active substances, but hold back the anaphylactinogenic ones.

The general procedure consisted in taking various tubercle bacillus preparations, the skin reactivity of which had been determined on tuberculous guinea pigs, and subjecting them to dialysis either in celloidin or in fish bladder bags, in



a closed system, testing the material outside the bag from time to time on tuberculous and normal guinea pigs. As soon as the material outside gave indication of containing the skin-reactive substance, the same material was used upon a sensitized and a normal uterus. In a few experiments we obtained results which at least indicate that the tuberculin active substance is more readily diffusible than the anaphylactinogens.

*Experiment 1.*—A glycerol salt solution extract of powdered tubercle bacilli was started on February 7, 1921. 1 gm. of powdered tubercle bacilli was infused in 500 cc. of a 5 per cent glycerol salt solution, heated to 70°C. for 1 hour, and placed in the incubator over night. It was then shaken for several hours on consecutive days. This material was simmered down to 150 cc. at a temperature of 75–80°, never quite being allowed to come to 80°, and was then filtered through paper. It gave a strong sulfosalicylic acid reaction. February 15. 40 cc. of this material

TABLE II.

*Readings on Skin Reactions Done February 17.*

Guinea pig No.	Skin reactions.	
	With material outside bag (B <sub>2</sub> ).	With material inside bag (B <sub>2</sub> ).
12	++	++++
13	++	++++
14	++	++++
15	—	++++
16	±	++
17	±	++
18	+	+++
Normal control.	—	±
" "	—	±

were placed in a fish bladder bag in a closed system with 40 cc. of salt solution outside. February 17. Skin reactions were done with the material inside and outside the bag, and it was found that a very definite though mild skin reaction resulted from the material outside the bag. The relative strength of the skin reaction with material inside and outside the bag are given in Table II.

February 18. This material was tested upon normal uteri and it was found that 2.5 cc. of the original material and the material outside the bag, added to a 200 cc. Ringer's bath in which a normal uterus was rhythmically contracting, produced no marked changes in the rhythm. The material was then tested on the uterus of a guinea pig sensitized as above described with tubercle bacillus extract. As will be seen in Fig. 7, 4 and 6 cc. of the material outside the bag, which gave a skin reaction, produced no spasm of the sensitized uterus, whereas 2 cc. from inside the bag gave a typical and marked reaction. We realize that this may have been a quantitative result, due to a greater sensitiveness of the skin than of the uterus.



This does not seem likely under the circumstances, but, nevertheless, must be considered.

We have noted above that Löwenstein and Pick claimed that they could completely free old tuberculin of its activity by prolonged dialysis against running water. For this reason, we determined to try the reverse experiment; that is, attempting by prolonged dialysis to remove the skin-reactive substance entirely from the inside of a dialyzing bag, leaving the anaphylactinogenic substance behind. We tried this with various preparations, but never succeeded even with prolonged dialysis in freeing the materials on the inside of the bag entirely of a skin-reactive substance, although such a result had been claimed by Löwenstein and Pick. In one case only did we obtain a result worth reporting. We placed 25 cc. of concentrated old tuberculin from the Department of Health of the City of New York in a fish bladder dialyzing bag, and dialyzed this against running water. The old tuberculin used still gave a powerful sulfosalicylic acid reaction for protein, and gave a definite precipitate on being acidified with acetic acid in the cold after dilution to about 1 : 20. The precipitate did not redissolve upon heating, but was rather intensified. With this preparation we had what we must now consider a rather fortunate accident. Because we were dialyzing with running water, we could not carry out the procedure in the refrigerator, and bacteria began to grow in the dialyzing bag. In the course of 5 days, the material from the inside of the bag gave practically no skin reaction on tuberculous guinea pigs as compared with old tuberculin diluted 1 : 3, which corresponded to approximately the dilution which had been attained by the substance in the course of dialysis. This material which no longer gave skin reactions was tested against normal and sensitized uteri, and it was found that, although 3 cc. of the material gave no reaction on the normal uterus, powerful reactions with sensitized uteri were obtained in quantities as low as 0.5 cc. and a weaker but still noticeable reaction obtained with 0.2 cc. The possibility that substances like histamine were formed by the bacteria and accounted for the uterine reaction can be excluded by the failure of this material to affect the normal uterus. Whatever may have been the reason for this result, whether dialysis had anything to do with it or whether it means simply that the bacterial growth had destroyed the tuberculin substances while leaving the anaphylactinogens intact, it seems a definite and sharp separation of the two functions.

### III.

#### *Difference between Infected Animals and Those Treated with Dead Bacterial Substances.*

Neither chemical fractionations nor the diffusion experiments furnish absolute proof that the anaphylactinogen can be completely separated from the tuberculin active substances. Nevertheless,

both series of experiments point in this direction, and, taken together with the mutual independence of the two types of reaction in guinea pigs, render such a conception an extremely likely one.

However this may eventually prove to be, one fundamental fact remains definite; namely, that the proteose fraction alone can elicit the particular form of hypersensitiveness which we speak of as the tuberculin reaction; and that the potency of the proteose residue in this respect is but slightly less than that of the whole extract, in spite of the fact that proteins have been removed as completely as is possible by boiling with acid.

Assuming, then, that the tuberculin type of reaction is a response to a proteose antigen, while the anaphylactic reaction is associated with the proteins, we are still confronted with the puzzling fact that the proteose reaction occurs only in infected animals and cannot ordinarily be induced in animals treated and rendered anaphylactic by injections of dead bacterial substances.

It might be assumed that the living tubercle bacillus in contact with the animal tissues produces a sensitizing substance which is not present in culture fluids or in dead tubercle bacilli. This idea is rendered improbable by the consideration that, although we almost uniformly fail in sensitizing guinea pigs to the cutaneous test by prolonged treatment with concentrated culture fluids or with bacillus extracts, nevertheless, skin reactions can be elicited in tuberculous animals by the proper application of such substances.

Another possibility, however, is that the substance which sensitizes to the tuberculin reactions is actually represented in the various tuberculin preparations and tubercle bacillus extracts, but that the intermittent method of injection, which must necessarily be used in the preparation of guinea pigs or other animals, does not simulate the manner in which these substances are being constantly diffused out into the animal tissues from growing foci. The supposition that the difference may lie in the manner of sensitization, both as to the time factor and in regard to the quantitative relations, is suggested by such experiments as those of Krause who has shown that cutaneous hypersensitiveness coincides with the establishment of a focus, diminishes with the healing of the focus, and varies directly with the intensity of the disease. It is suggested that in specific phenomena

of hypersensitiveness, of the tuberculin, typhoidin, mallein types, etc., we may be dealing with antigens that are subject to laws of sensitization and antibody formation, quite different from those governing the phenomena of protein anaphylaxis; in which the hypersensitiveness is elicited only by an intensive and concentrated contact with the antigenic substances; and in which, soon after the stimulus is removed, the hypersensitiveness diminishes. This, at least, seems a logical conclusion in regard to guinea pigs.

It should also be borne in mind, as has been suggested by Krause, that a general tuberculin reaction blunts skin hypersensitiveness considerably, and that a similar blunting of both the anaphylactic and the skin hypersensitiveness has been noted by others, as well as by ourselves, in the late stages of a fatal tuberculosis in guinea pigs, observations which indicate a reaction at least analogous to antigen-antibody reactions in general.

These considerations suggested to us the possibility that, although we might be giving the animals which we had treated with bacterial extracts some of the material which sensitizes them in the course of infection, we were not, perhaps, administering to them a sufficient amount of this proteose-like substance and were not giving it with the continuity with which it passes into the circulation of an animal suffering from an active process.

In order to obtain some light upon this, we carried out a number of experiments as follows:

Tubercle bacillus cultures were grown on 5 per cent glycerol peptone broth. When the growth had reached the size of a silver dollar or slightly more, but one-quarter of this growth was carefully lifted into another flask containing 100 cc. of similar glycerol broth, and the remaining three-quarters was killed at 80°C. for  $\frac{1}{2}$  hour. This dead growth, at least three-quarters of the original growth, was now washed several times with salt solution and infused in a flask containing 100 cc. of broth of the same lot as that on which the living one-quarter had been inoculated. The two flasks were then put into the incubator, and after the 2nd day skin reactions were done every day with the fluids from these flasks on tuberculous guinea pigs.

Fig. 8 shows the results. The reaction marked 1 on this figure is the reaction obtained with 0.1 cc., intracutaneously injected, of a 1:3 dilution of the 4 day growth in a highly sensitive guinea pig. The

reaction marked 2 shows the corresponding reaction carried out with the broth in which the dead tubercle bacilli, three times greater in bulk than the living ones, had been infused for the same length of time and used in the same dilution.

In order to eliminate any obstruction to diffusion which might have been brought about in the dead culture by the fact that heat was used in the killing, we did other experiments in which 0.5 per cent carbolic acid was added to the culture material to kill it.

Such an experiment is the following one. In this case a living growth about 1.2 cc. in diameter was floated on a flask of 100 cc. of glycerol broth and the remainder, about four times this amount, was submerged by shaking in 100 cc. of a similar broth flask, and 0.5 per cent carbolic acid added. On the 6th day reactions were done on tuberculous guinea pigs and on a control with the results shown in Table III.

TABLE III.

*Skin Reactions Obtained with Material Killed with Carbolic Acid.*

Broth.	Dilution.	Results with tuberculous guinea pigs.			Results with normal control.
		No. 19.	No. 20.	No. 21.	
From living bacilli.	1:10	+	++	++	—
	1:20	+	+	±	—
From killed bacilli.	1:10	+	+	—	—
	1:20	±	±	—	—

Reactions of this type would indicate that the materials which caused skin reactions of the tuberculin type were being constantly diffused out from the growing cultures, while a limited amount only could be extracted from dead tubercle bacilli.

It is not impossible also that such a conception might indicate that these proteose-like materials were constantly being produced from the more complex material which we have spoken of tentatively as nucleoproteins, which represent the bulk of the soluble bacterial constituents and that this is the reason why we have never been able to free these acid-precipitable substances of their skin reaction capacity.

With this in mind we have recently treated four guinea pigs with large amounts of the so called nucleoproteins, giving each animal

four intraperitoneal injections, each of which represented at least 50 cc. of original extract. And in these animals, 14 days after the last injection, we saw the first hopeful indications of positive skin reactions of the tuberculin type, artificially induced. These experiments will be continued.

#### IV.

##### DISCUSSION AND CONCLUSIONS.

The work reported in the preceding sections justifies, we think, a number of definite conclusions. In addition to this, some of the experiments indicate a line of thought which may lead to considerable alteration in our conceptions, both of phenomena of bacterial hypersensitiveness and of infection.

1. In guinea pigs two fundamentally different types of intradermal reactions may be observed. One of these is the immediate, transitory reaction which develops in animals sensitized against proteins (horse serum, etc.) and may be regarded as one of the manifestations of general protein hypersensitiveness, or anaphylaxis; the other is the tuberculin type of skin reaction which develops more slowly, leads to a more profound injury of the tissues and is independent of anaphylaxis as ordinarily conceived.

2. The tuberculin type of hypersensitiveness (as well as probably the typhoidin, mallein, abortin reactions, etc.) does not develop at all in guinea pigs sensitized with proteins, like horse serum, etc. While this form of hypersensitiveness may eventually be induced with materials not bacterial in origin, it has been observed up to date only as a reaction of bacterial infection.

3. Methods of treatment with protein material from bacterial cultures which sensitize guinea pigs to anaphylactic reactions with the bacterial extracts, do not sensitize them to the tuberculin type of reaction. Such sensitization is easily accomplished only by infecting the animals with living organisms. No reliable method of sensitizing guinea pigs to such reactions with dead bacterial material has as yet been worked out, though a few hopeful experiments have been obtained with massive injections of large amounts of the acid-precipitable substances (nucleoproteins?) from bacterial extracts.

4. In animals made hypersensitive to the tuberculin type of reaction by infection with living bacteria, the reaction may be elicited by intradermal injections of bacterial extracts from which all coagulable proteins, nucleoproteins, and Bence-Jones proteins have been removed, as well as this can be done by boiling with acid, etc. This proteose residue alone suffices to elicit such reactions. The exact chemical nature of the so called proteose residue must be further studied and analyzed when we have had opportunity to produce bacterial extracts in large quantity.

These points seem incontrovertible on the basis of our own experiments, as well as those of other workers.

There thus seem to develop two definite forms of hypersensitivity in guinea pigs infected with bacteria, typical anaphylaxis in which the protein material of the bacterial cells is concerned, which develops late and which can be induced by repeated injections of dead bacterial material, and a hypersensitivity to non-protein constituents which differs from the former, both in the laws that govern sensitization and in the manifestations which follow injections into the sensitized animals.

While there is virtual agreement among immunologists concerning the essential mechanism of protein anaphylaxis, its dependence upon an antigen-antibody reaction, and the dominating rôle played by the sessile antibodies, the mechanism of hypersensitivity to tuberculin and similar bacterial substances is still a problem of much uncertainty.

The most striking difference between the two phenomena lies, as we have seen, in the criteria of sensitization, in that hypersensitivity to the tuberculin type of reaction can hardly ever be induced by any of the ordinary methods of preparation with the constituents of dead bacteria, but develops promptly (7 to 10 days) in the course of actual infection with living organisms.

The considerable specificity of such reactions forces the conclusion that the sensitizing substance must, in some way, be derived from the infecting microorganisms.

The idea that the failure of sensitization with dead culture materials is perhaps due to the elaboration in the body of infected animals of bacterial products not represented in extracts of test-tube cultures is rendered unlikely by the fact that in the tuberculin-sensitive,

infected animals, we can produce the reactions by the application of such dead extracts. It is neither logical nor in keeping with biological experience to assume that one substance will sensitize to reaction with another. This mistake was made early in the study of anaphylaxis in another connection and caused considerable delay of progress.

Krause has shown that tuberculin sensitiveness may be blunted in infected animals by massive, but sublethal injections of tuberculin, and we have obtained some indications of the same thing. Moreover, others as well as ourselves have seen tuberculin reactivity decline in guinea pigs and in man in the stages of very severe infection. These facts would eliminate any assumption of mere cumulative injury as explaining this type of reaction, and stamp it as a mechanism at least analogous to ordinary anaphylaxis.<sup>7</sup>

The only remaining possibility to explain the difference between infected animals and those treated with dead bacterial constituents would be to assume that the difference must lie in the manner in which the sensitizing substance is administered to the animals, and that sensitization with the proteose residue materials depends upon criteria of sensitization differing in regard to the time and quantity factors from those governing protein sensitization. If one considers the relatively simpler chemical structure and perhaps physically greater diffusibility of the materials concerned in this reaction, one might readily expect such differences in the methods needed for sensitization.

In keeping with such a line of reasoning our experiments have shown that the tuberculin active materials are constantly and rapidly being diffused out into the culture fluid from growing organisms, in quantities greater than can be extracted from similar amounts of the dead bacteria. It seems reasonable to assume from this that the same thing may happen in the animal body harboring a growing focus. And it would seem quite likely that the association of the tuberculin type of reaction with actual infection may depend upon the fact that sensitization to these non-protein substances depends upon a constant steady absorption of large amounts of the material.

Moreover, the only hopeful experiments on the artificial production of tuberculin sensitiveness in guinea pigs obtained by us were those

<sup>7</sup> Direct attempts to show such cumulative toxic action have failed in our hands.



in which massive doses of the nucleoprotein material injected into guinea pigs gave rise to a moderate skin sensitiveness.

Does the so called proteose residue form antibodies, and, if so, are substances analogous to antibodies involved in the tuberculin type of hypersensitiveness?

The failure to transfer passively this form of hypersensitiveness to normal animals with the blood and tissues of tuberculin-sensitive ones would suggest that no antibodies are involved. But this is not conclusive on the basis of available experimental facts. We are inclined to believe that antibodies of a sort are involved, for the following reasons: (a) In our experiments with the uteri of highly sensitive extract-treated guinea pigs and of tuberculous guinea pigs, we have occasionally had positive reactions when the proteose residue alone was used. (b) We believe that these proteose substances are entirely analogous to the substances studied by Avery and Dochez (22) in the urine and blood of typhoid and pneumonia patients. They obtained precipitin reactions against homologous immune sera with the urine of infected cases concentrated by evaporation after boiling with acetic acid to remove coagulable proteins. (c) Petroff, with whom we discussed this proteose residue early in our work, has produced it, and tells us that he has obtained precipitin reactions with it by titrating it against the serum of a sheep treated for a long time with tubercle bacillus products.

In suggesting an antibody response to a non-protein antigen we are aware that we are opposing what has been regarded as a well established doctrine in immunity; this is justified, or at least mitigated, we believe, by the consideration that reactions of the antigen-antibody type are the only explanation of specificity; and tuberculin, mallein, and typhoidin reactions are to a considerable degree specific. If such reaction bodies cannot be produced by precisely the same methods of administration as to time and quantity which are successful in calling forth protein antibodies, this should not astonish us, since, after all, the substances that we are dealing with are simpler in chemical structure than are the proteins, and physically are probably of relatively greater diffusibility. It may be that the greater diffusibility of the proteose-like substances transfers much of the actual reaction phenomena to an intracellular location, and that this



to some extent influences the presence of circulating antibodies. It may also be that these more diffusible non-protein antigens are more rapidly eliminated from the animal body than are the proteins. Indeed, the above mentioned observations of Avery and Dochez, and the recent work of Wildbolz (23), Lanz (24), Imhof (25), and Gibson and Carroll (26), who demonstrated tuberculin active antigens in the urine of active cases, would corroborate such a view. The evidence available at the present time, however, concerning antibody formation to these non-protein substances is, we recognize, largely indirect, at least as far as our own work is concerned, and we present it in the present connection purely as a working hypothesis.

Finally, perhaps the most important theoretical consideration indicated by our experiments is the following. We have in the tuberculin reaction a form of hypersensitiveness which seems to be (in guinea pigs, at least) analogous entirely to the typhoidin reaction, the mallein reaction, and the abortin reaction. Whenever reactions of this type have been carefully studied, whatever the bacteria involved, they have been associated with infection as in tuberculosis, and have been followed by analogous clinical manifestations. It would seem perhaps that we are dealing with a law applicable to bacterial infection in general.

It would appear that certain non-coagulable substances of uncertain chemical constitution are being constantly elaborated in the course of bacterial growth, and passed into the circulation of infected animals. As a result of this, infected animals become sensitized to these heat- and acid-resistant materials, in tuberculosis in the course of 1 to 2 weeks, in the case of more rapidly growing bacteria perhaps sooner. Early in the course of infection, the animal becomes sensitized and subsequently the further elaboration and distribution of these materials from the bacterial focus plays a fundamental part in the injury of the animal. These proteose-like substances, like tuberculin, possessing but slight toxicity for the normal animal, become highly toxic to the sensitized one. Thus, these substances, while not being true exotoxins in the ordinary sense, would still represent a highly toxic bacterial product comparable in its injurious effect to toxins when produced in the body of an animal thus sensitized.

If there is any value in these deductions the attention of bacteriologists should be turned to the non-protein constituents of bac-

terial cells in their further immunological studies, as well as to the protein materials.

It is obvious that the next step in our investigations must consist in producing the non-coagulable material from bacterial extracts in considerable quantity, to determine their antibody-forming properties in detail, and elucidate, if possible, the laws which govern sensitization with them. This work has been begun, but it has seemed advisable to publish this as far as we have gone because it will take a long time before it can be completed.

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## EXPLANATION OF PLATES.

## PLATE 40.

FIG. 1. Guinea Pig 22. Another instance of positive skin reaction after 9 days, with negative uterine test. This animal was of the same lot as Guinea Pig 23.

FIG. 2. This record is a simultaneous record of two guinea pigs inoculated on November 21, 1920, with large amounts (2 cc.) of a suspension of a young tubercle bacillus culture (human). No. 23 (upper curve) inoculated intraperitoneally, and No. 24 (lower curve) subcutaneously. On December 1, 1920, skin reactions were done with two types of extract, one a sodium carbonate extract and the other a sodium hydroxide extract. No. 23, which was apparently weaker and emaciated, showed practically no reaction in either place, whereas No. 24, to our surprise, showed a definite reaction at both points. In spite of the differences in skin reactions, it will be seen that the uterine reactions in both guinea pigs were negative. The two uteri were put into the same bath simultaneously in order to subject them to exactly the same conditions. The negative reaction of the uterus was confirmed with the other horns of both guinea pigs. The surprising thing is the earliness with which the skin reaction appeared, which tends to confirm one of a series of observations that the skin reaction appears much earlier than the uterine reaction.

FIG. 3. Guinea Pig 25. Inoculated subcutaneously with tubercle bacilli on November 22, 1920. Showed that powerful anaphylactic reaction may develop within 3 weeks after a heavy inoculation with tubercle bacilli. This, however, is unusually early. Skin reaction in this guinea pig was positive.

## PLATE 41.

FIG. 4, *a* and *b*. (*a*) Guinea Pig 26. (*b*) Guinea Pig 27. Two guinea pigs of a series injected every other day intraperitoneally with unfiltered alkaline extract of powdered tubercle bacilli. Last injection given November 29, 1920. No. 26 gave a negative skin reaction on December 6, but a positive uterine reaction on December 7. A similar uterine test with another guinea pig (No. 28) showed a very moderate uterine reaction, and this, with other experiments, indicates that the uterine or anaphylactic reaction does not develop until at least 8 or 9 days after the last preparatory injection. Guinea Pig 27 in the second record was treated exactly like the preceding animal, but here the tests were not done until December 21, slightly over 3 weeks since the last extract injection. Here again the skin reaction was entirely negative and the uterine test very much more powerful. The uterine reaction does not develop to its fullest extent until about 2 or 3 weeks after the last injection.

FIG. 5, *a* to *c*. (*a*) Normal guinea pig. (*b*) Guinea Pig 29. (*c*) Guinea Pig 30. The records show a failure of reaction of the uteri of two different sensitized guinea pigs (Nos. 29 and 30) after the addition of the proteose residues alone, but

powerful reactions when, following this, whole extracts were introduced into the bath. The record at the top is a normal control with the same extracts, showing that the reactions below are specific.

PLATE 42.

FIG. 6, *a* and *b*. This is one of two preparations in which the materials used consisted of two fractions obtained from the same 50 cc. of a 0.02 per cent alkaline extract of powdered tubercle bacilli. The extract was centrifuged until clear, the supernatant fluid acidified to pH 4.5, which was the point at which the flocculent precipitate did not increase. This was centrifuged away and spoken of as nucleoprotein. This nucleoprotein was twice redissolved in more than 50 cc. of alkaline salt solution and reprecipitated with acid, the material being centrifuged in each case in order to purify as much as possible. The supernatant fluid was then boiled in the acid condition to remove coagulable proteins and twice filtered through a Berkefeld filter. It will be noted that in looking at these two preparations together after testing against different strips of the same sensitized guinea pig uterus (No. 31), the total amounts of the two fractions gave the same results, though in different proportions, an additional proof that cumulative effects had nothing to do with the results.

FIG. 7. February 18. The sensitized guinea pig treated with Material B<sub>2</sub> which, as stated before, gave definite though somewhat weakened skin reactions. Here, 6 cc. on the outside of the bag, which still gave skin reactions, gave no anaphylactic reaction. The high rise is probably due to the fact that this uterus was very irritable. That the rise was not an anaphylactic reaction is apparent from the immediate return to the base-line, and the fact that there was no apparent desensitization to the subsequent addition of 2 cc. from the inside of the bag. Dialysate was positive on skin and negative on uterus.

PLATE 43.

FIG. 8. Drawing of skin reactions on a tuberculous guinea pig, carried out with broth from a culture of living tubercle bacilli 4 days old, 1:3 dilution, at the point marked 1, and a similar reaction with a similar amount of broth from a killed culture, 1:3 dilution, at the point marked 2.

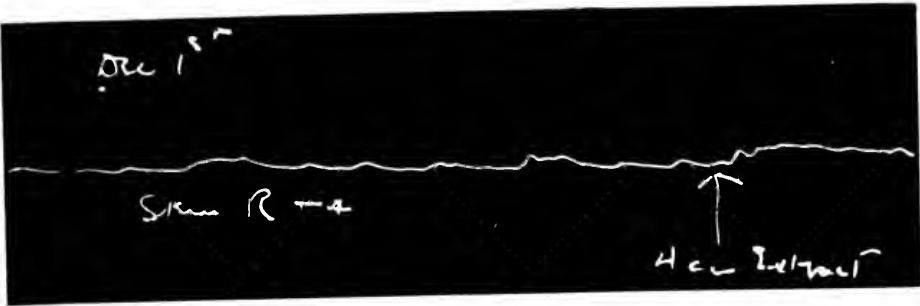


FIG. 1.

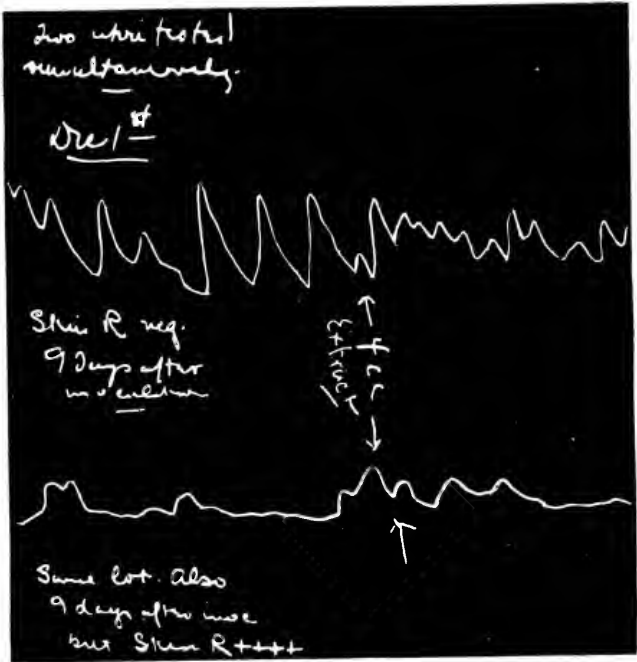


FIG. 2.

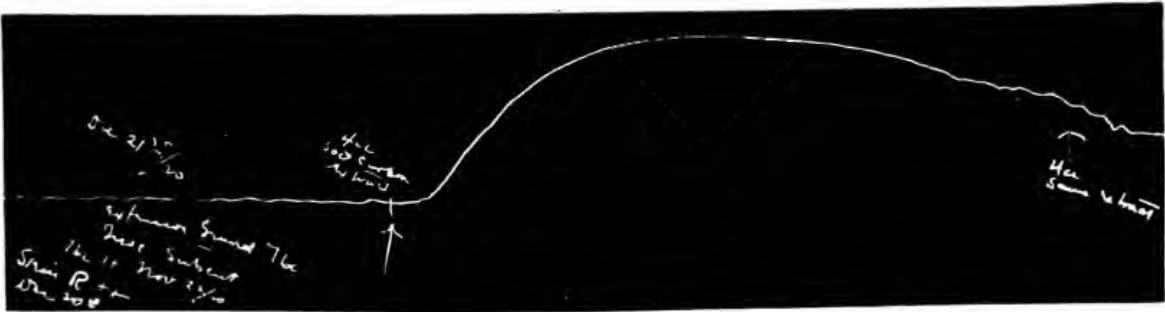


FIG. 3.

(Zinsser: Tuberculin reaction.)



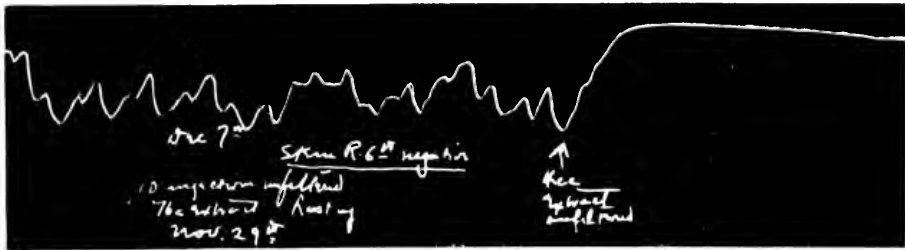


FIG. 4, a.

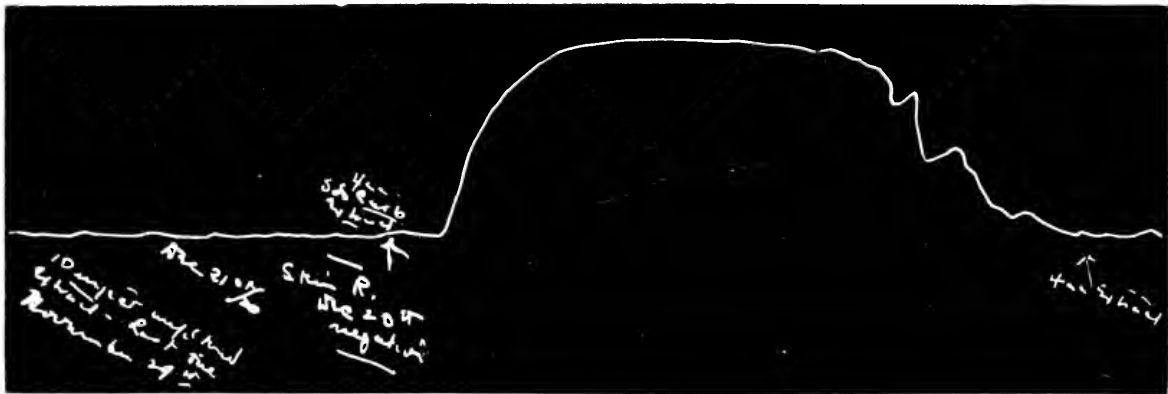


FIG. 4, b.

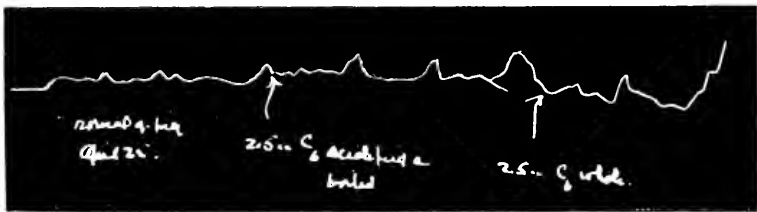


FIG. 5, a.

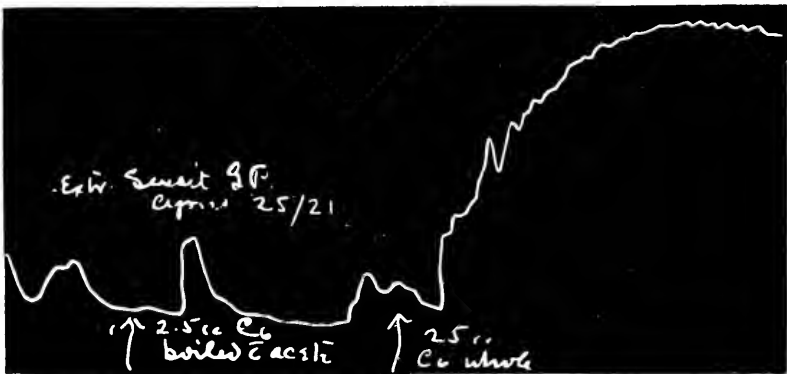


FIG. 5, b.

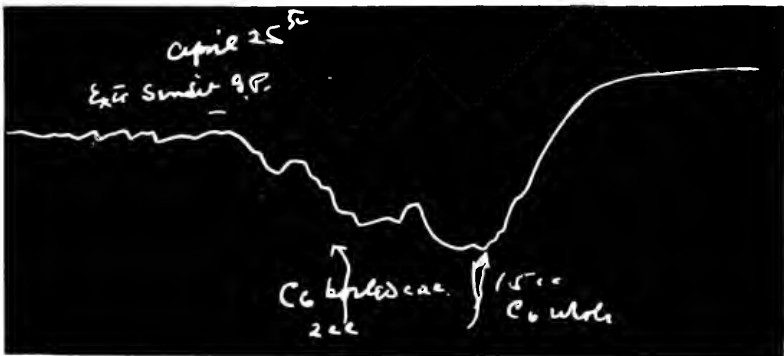


FIG. 5, c.





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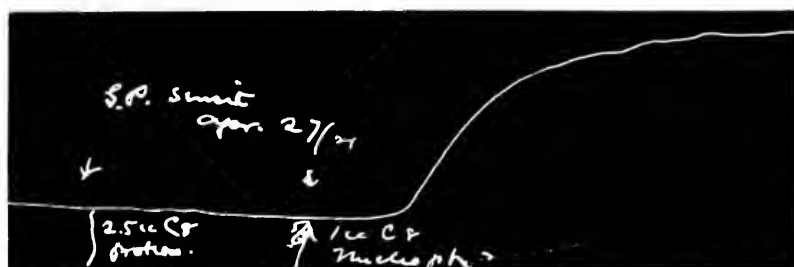


FIG. 6, a.

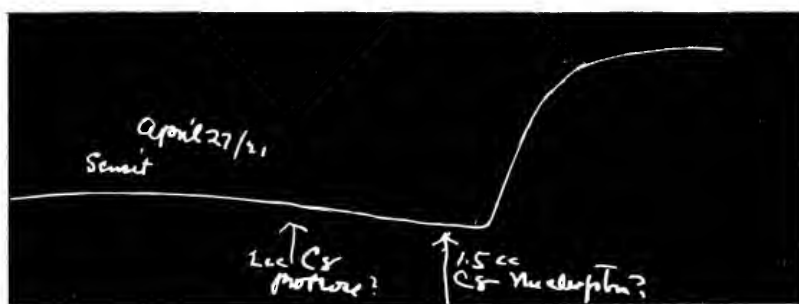


FIG. 6, b.

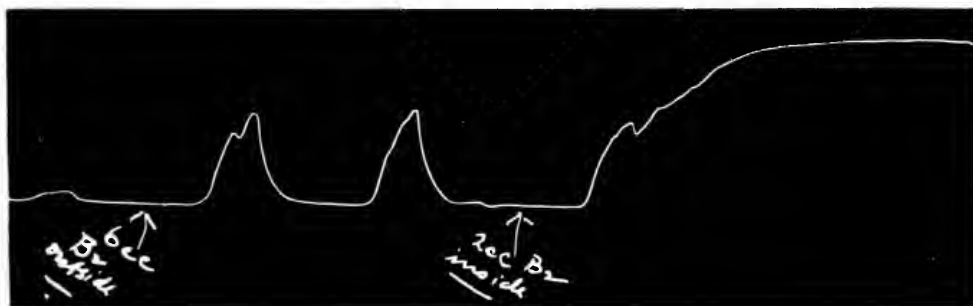


FIG. 7.

(Zinsser: Tuberculin reaction.)



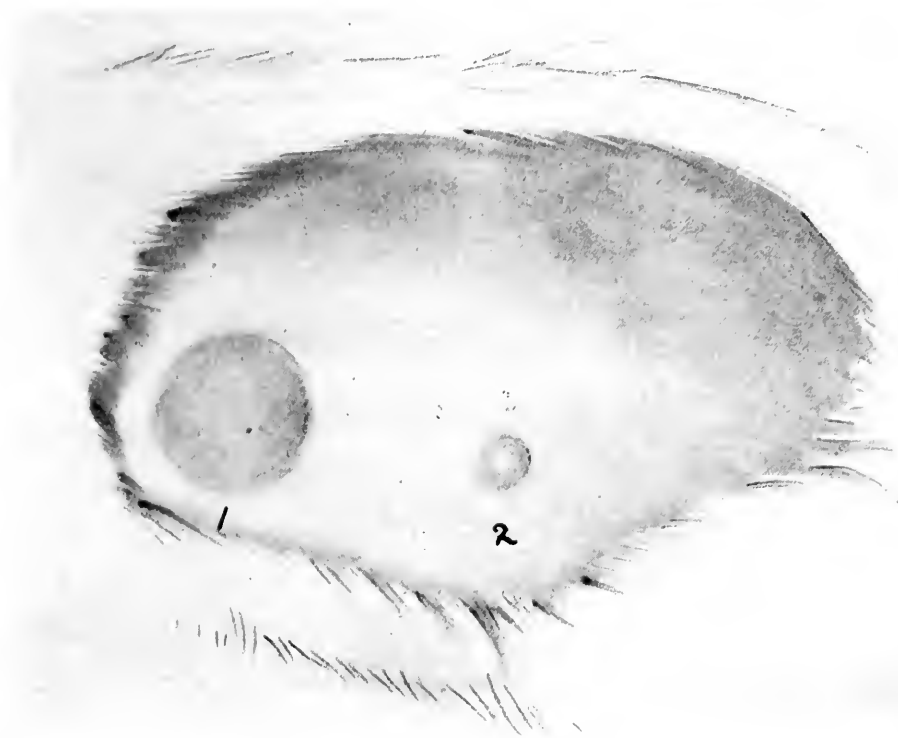


FIG. 8.

(Zinsser: Tuberculin reaction.)



# EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

## I. CONCURRENT INFECTIONS DURING THE COURSE OF EXPERIMENTAL TYPHUS FEVER IN GUINEA PIGS.

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### INTRODUCTION.

The past 12 years have witnessed an energetic investigation into the precise nature of the virus of typhus fever. From the earlier work of Nicolle and his associates, Anderson and Goldberger, and Ricketts and Wilder, to the recent efforts of a number of other investigators, the exact pathology of the disease in man and in the experimental animal has been defined, the carrier of the virus and the mode of its spread have been determined, and our knowledge of the action of the virus has been amplified.

During this period a number of bacteria have coincidentally been brought forward as the inciting agent of the disease; and the two for which certain proofs of relationship to typhus fever have been presented are the *Rickettsia prowazeki* of da Rocha-Lima<sup>1</sup> and the *B. typhi exanthematici* of Plotz.<sup>2,3</sup>

The writer was led several years ago to the acceptance of the etiological relationship of Plotz' bacillus to typhus fever by the fact that he found specific antibodies against the organism in the blood of typhus patients;<sup>4</sup> that with it, it was believed, experimental typhus in guinea pigs had been induced<sup>4</sup> and that a similar bacterium was recovered from typhus-infected lice.<sup>5</sup>

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<sup>2</sup> Plotz, H., *J. Am. Med. Assn.*, 1914, lxii, 1556.

<sup>3</sup> Plotz, H., *Presse méd.*, 1914, xxii, 411.

<sup>4</sup> Plotz, H., Olitsky, P. K., and Baehr, G., *J. Infect. Dis.*, 1915, xvii, 1.

<sup>5</sup> Olitsky, P. K., Denzer, B. S., and Husk, C. E., *J. Infect. Dis.*, 1916, xix, 811.

With reference to the presence of specific antibodies, it was generally believed at the time the work was done that their demonstration was of the utmost importance in establishing an etiological relationship between an organism and the disease in which such antibodies were found. But during the 5 years which have passed, increasing knowledge has shed new light on the significance of specific antibodies and the nature of the typhus virus. Wilson<sup>6</sup> demonstrated that blood of typhus patients readily agglutinated intestinal, colon-like bacilli. More recently, Weil and Felix<sup>7</sup> recovered *proteus* bacilli, termed by them *B. proteus* X<sub>19</sub>, from the urine of typhus patients which not only were agglutinated by the serum from these cases but also were agglutinated to an even higher degree than Plotz' bacillus. Furthermore, the agglutination with *B. proteus*, or, as this is commonly designated, the Weil-Felix reaction, corresponds not infrequently to an immunity reaction; that is, the agglutinins appear late in the disease, increase in concentration at the crisis, and persist for months (Oettinger,<sup>8</sup> Sampietro,<sup>9</sup> and others). Complement-fixing, bacteriolytic as well as precipitating antibodies against the *proteus* bacilli have also been observed in the blood of typhus patients.<sup>10</sup> Agglutinins were subsequently found for still other organisms. For example, Neukirch and Kreuscher<sup>11</sup> isolated from the stools of typhus patients a strain of *B. pyocyaneus* which was agglutinated by the serum of typhus patients in a manner resembling the agglutination of *B. proteus*. Fikai<sup>12</sup> and others noted a similar reaction with *Micrococcus melitensis*. Werner and Leoneanu<sup>13</sup> and Sampietro<sup>9</sup> observed agglutinins for the typhoid bacillus.

Hence the question arises as to the significance of the antibodies against Plotz' bacillus occurring in the blood of typhus patients.

<sup>6</sup> Wilson, W. J., *J. Hyg.*, 1909, lx, 332; 1910, x, 155.

<sup>7</sup> Weil, E., and Felix, A., *Wien. klin. Woch.*, 1916, xxix, 974.

<sup>8</sup> Oettinger, W., *Centr. Bakt., 1te Abt., Orig.*, 1918, lxxx, 304.

<sup>9</sup> Sampietro, G., *Ann. ig.*, 1920, xxx, 593.

<sup>10</sup> The literature on this subject is extensively reviewed by Zlocisti, T., *Ergebn. Hyg.*, 1920, iv, 100.

<sup>11</sup> Neukirch, P., and Kreuscher, A., *Beitr. Klin. Infektionskrankh.*, 1920, viii, 68.

<sup>12</sup> Fikai, G., *Ann. ig.*, 1920, xxx, 395

<sup>13</sup> Werner, H., and Leoneanu, E., *Münch. med. Woch.*, 1918, lxxv, 587.

With regard to the second point, that experimental typhus fever can be induced with *Bacillus typhi exanthematici*, doubt is cast upon this belief by later studies. As we have indicated in a previous paper,<sup>14</sup> four requisites determine experimental typhus fever: (a) induction of the typical disease through inoculation from animal to animal, indefinitely; (b) presence of the characteristic vascular lesions, especially in the brain; (c) absence of secondary infections with ordinary bacteria; and (d) development in recovered animals of immunity to subsequent injections of typhus virus. All these requirements should be fulfilled in a given case, since Friedberger<sup>15</sup> has shown that non-specific fevers which simulate experimental typhus fever can occur in guinea pigs. The four requirements have not been met in the experiments with the Plotz bacillus.

In respect to the third point, namely the isolation of an organism similar to the Plotz bacillus from typhus-infected lice, the subsequent demonstration of rickettsia bodies in this insect involves the question of their identity. From a purely morphological comparison, the assumption was made that *Bacillus typhi exanthematici* was identical with *Rickettsia prowazeki* of da Rocha-Lima;<sup>5</sup> but the latter bodies have thus far resisted all efforts at artificial cultivation, including even the method of Plotz.<sup>16</sup>

In this paper we shall describe the occurrence of concurrent infections among guinea pigs in the line of transmission of human and louse strains of a Polish typhus fever virus, which we obtained through the kindness of Dr. S. B. Wolbach, and of another human strain, which we have recovered from a Czecho-Slovak immigrant recently arrived at the Port of New York. These strains, described in a previous communication,<sup>14</sup> have been shown to be identical by cross-immunity tests.

#### *Method.*

The three strains of typhus virus were propagated in guinea pigs by means of the intraperitoneal injection of 3 cc. of the blood from an antecedent affected animal. The blood was obtained by cardiac

<sup>14</sup> Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

<sup>15</sup> Friedberger, E., *Z. Immunitätsforsch., Orig.*, 1920, xxix, 125.

<sup>16</sup> Schultz, E. W., *Am. J. Med. Sc.*, 1921, clxi, 78.

puncture<sup>17</sup> with a sterile needle and syringe previously washed in 50 per cent sodium citrate solution. Immediately before the next series of normal guinea pigs was injected with this blood, it was inoculated into various media, including the Plotz medium employed for the cultivation of *Bacillus typhi exanthematici*,<sup>4</sup> the Smith-Noguchi anaerobic tissue medium,<sup>18</sup> human ascitic fluid with or without petrolatum, horse serum diluted 1:3 with saline solution, and 1 per cent dextrose broth with or without rabbit blood. In addition to the blood, the splenic tissue of the guinea pigs, cut in small fragments, was likewise subjected to cultivation in the same kinds of media.

### *Results of Cultivation.*

The blood and splenic tissue of 58 guinea pigs in the line of transmission of the three strains of typhus virus were thus investigated. Different bacteria were found in 24 of the animals, either in the blood or in the spleen or in both. Table I summarizes the kinds of bacteria cultivated and the number of guinea pigs in which they were found.

TABLE I.  
*Kinds of Bacteria and Frequency of Occurrence.*

Kind.	No. of guinea pigs in which bacteria were found.	
	Spleen.	Blood.
<i>B. typhi exanthematici</i> Plotz.....	3	5
Anaerobic streptococcus.....	0	5
<i>Staphylococcus aureus</i> .....	0	4
Gärtner type bacillus.....	2	2
Aerobic diphtheroid.....	2	0
<i>B. proteus</i> .....	0	2
" <i>welchii</i> .....	1	0
Aerobic Gram-positive diplobacillus.....	0	1

As the table shows, various bacteria were found in the cultures in 24, or 41 per cent, of the guinea pigs studied during the

<sup>17</sup>Chloroform anesthesia was given the animals during the operation. Chloroform was employed instead of ether in order to prevent explosions while working near a Bunsen flame.

<sup>18</sup>Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.



course of experimental typhus fever. The more frequent among these is an organism indistinguishable from *Bacillus typhi exanthematici* of Plotz. In two animals, however, this organism was found in the blood but not in the spleen, which yielded pure cultures of Gärtner type bacilli; in a third, the Plotz bacillus was found in the blood in conjunction with an anaerobic streptococcus. Finally, three guinea pigs which showed the Plotz organism in the spleen did not show it in the blood.

The lack of regularity of occurrence of any of these microorganisms is further emphasized by an analysis of the stage of experimental typhus fever in which they were encountered. Thus the blood of the guinea pigs in the line of transmission of the typhus virus was taken and cultured during the period of incubation, and on the 1st, 2nd, 3rd, and 4th days of the fever.

*Blood Cultures during the Incubation Period.*

Nicolle and Blaizot<sup>19</sup> have demonstrated that the blood of a monkey injected with typhus virus is infective for other animals 48 hours before the onset of fever; Doerr and Pick<sup>20</sup> showed that guinea pigs are infective 2 to 4 days after inoculation with virus. Our own experience indicates that the blood of inoculated guinea pigs is infective 4 days prior to the onset of fever, or about 3 to 5 days after inoculation. In any event, the material used in the experiments summarized in Table II sufficed to induce typical experimental fever on injection into normal guinea pigs. It will be noted from this table that the blood of all the guinea pigs, seven in number, taken on the 6th to 10th days after inoculation and before the onset of fever,<sup>21</sup> failed to yield on culture any of the bacteria mentioned in Table I. Yet this blood was virulent, for in each instance it was capable of reproducing in guinea pigs typical experimental typhus fever.

<sup>19</sup> Nicolle, C., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1916, ix, 127.

<sup>20</sup> Doerr, R., and Pick, R., *Wien. klin. Woch.*, 1918, xxxi, 829.

<sup>21</sup> In a series of over 100 guinea pigs, inoculated with typhus virus, which we have observed, the incubation period varied from 5 to 19 days, averaging 9 to 10 days. The febrile period, or continued temperature of 40°C., or higher, varied from 4 to 9 days, averaging 6 to 7 days.

In other words, typhus virus taken before the onset of fever in the experimental disease is free from admixture with any ordinary bacteria.

TABLE II.  
*Blood Cultures during the Incubation Period.*

Period of incubation.	Results of culture.
days	
9	No growth.
9	" "
9	" "
10	" "
6	" "
9	" "
9	" "

*Cultures of Blood and Spleen on the 1st Day of Fever.*

In a series of twenty-six guinea pigs, cultures of the blood and spleen were made on the 1st day of fever. Table III summarizes the results of these cultures. The table shows that the conditions met with during the period of incubation of the virus have undergone a change. In six of the guinea pigs, or 23 per cent, the active blood or spleen, or both, yielded different kinds of bacteria. With the blood itself typical experimental typhus fever could be reproduced by transmission,<sup>22</sup> excepting with that containing *Bacillus proteus*, although the characteristic lesions were found in the antecedent animals. Of the different bacteria encountered, the Plotz bacillus was more often met with than the others. The remaining twenty animals yielded none of these bacteria.

In the animals in which the Gärtner type bacilli, *Bacillus proteus*, and the staphylococci were found, the spleen was very much enlarged and was covered with a fibrinous exudate, while the remaining viscera and peritoneum were deeply congested. In the animals in which the other bacteria were encountered, the pathological picture was indistinguishable from that of typical experimental typhus fever.<sup>14</sup>

<sup>22</sup> In event of the virus showing ordinary bacteria, it can usually be freed from them by subcutaneous instead of intraperitoneal inoculation of guinea pigs.

TABLE III.  
*Cultures of Blood and Spleen on the 1st Day of Fever.*

Period of incubation. days	Bacteria recovered.	
	Spleen.	Blood.
7	None.	None.
8	"	"
12	"	"
10	"	Anaerobic streptococcus.
11	"	<i>B. typhi exanthematici</i> Plotz.
7	"	None.
7	Aerobic diphtheroid.	"
9	None.	"
5	"	<i>B. proteus</i> .
8	"	None.
8	"	"
14	Gärtner type bacillus.	<i>B. typhi exanthematici</i> Plotz.
7	None.	None.
9	"	"
6	"	"
9	"	"
19	"	"
9	"	"
11	"	"
9	"	"
6	"	"
6	"	"
10	"	"
7	"	<i>Staphylococcus aureus</i> .
8	"	None.
8	"	"

*Cultures of Blood and Spleen on the 2nd Day of Fever.*

In a series of sixteen guinea pigs, cultures of the blood and spleen were made on the 2nd day of the febrile reaction. The results of these cultures are given in Table IV. Table IV shows that the variety and number of bacteria encountered in association with the active blood and spleen become larger, in proportion to the duration of the fever. Indeed, 10, or 62.5 per cent, of the guinea pigs on the 2nd day of the fever yielded from the blood or spleen, or both, different bacteria, among which the Plotz bacillus and anaerobic streptococci were the ones most frequently found.

In this series, as in the previous one, the guinea pigs of which the blood or spleen yielded growths of staphylococci, bacilli of the Gärtner type, and Welch's bacilli showed distinctive pathological effects such as fibrinous splenitis and congestion of the other organs; while those in which the other bacteria were found, exhibited merely the lesions of experimental typhus fever.

TABLE IV.

*Cultures of Blood and Spleen on the 2nd Day of Fever.*

Period of incubation. days	Bacteria recovered.	
	Spleen.	Blood.
9	None.	None.
5	"	"
10	"	<i>B. typhi exanthematici</i> Plotz.
8	"	" " " "
8	"	None.
6	"	"
6	Gärtner type bacillus.	Gärtner type bacillus.
6	<i>B. typhi exanthematici</i> Plotz.	None.
7	None.	"
10	"	Anaerobic streptococcus.
10	<i>B. welchii</i> .	<i>Staphylococcus aureus</i> .
6	None.	<i>B. typhi exanthematici</i> Plotz and anaerobic streptococcus.
12	Aerobic diphtheroid.	None.
11	None.	Anaerobic streptococcus.
18	"	Aerobic Gram-positive diplobacillus.
5	"	None.

*Cultures of Blood and Spleen on the 3rd Day of Fever.*

In a series of four typhus guinea pigs cultures of the blood and spleen were made on the 3rd day of the fever. The results of these cultures are tabulated in Table V which shows that on the 3rd day of the fever three of the four animals yielded on cultivation of the blood and spleen a variety of bacteria, among which were *Bacillus typhi exanthematici* of Plotz, Gärtner type bacilli, and anaerobic streptococci.

TABLE V.

*Cultures of Blood and Spleen on the 3rd Day of Fever.*

Period of incubation.	Bacteria recovered.	
	Spleen.	Blood.
<i>days</i>		
11	<i>B. typhi exanthematici</i> Plotz.	None.
7	Gärtner type bacillus.	Gärtner type bacillus.
11	None.	Anaerobic streptococcus.
9	"	None.

*Cultures of Blood and Spleen on the 4th Day of Fever.*

In Table VI a résumé is given of the results of the cultures of the active blood and spleen made from four guinea pigs on the 4th day of the experimental disease. These cultures yielded from the blood and spleen bacteria, among which *Staphylococcus aureus* was found in the blood and spleen of two animals, Plotz' bacillus in the spleen but not in the blood of the third animal, and *Bacillus proteus* in the blood only of the fourth.

TABLE VI.

*Cultures of Blood and Spleen on the 4th Day of Fever.*

Period of incubation.	Bacteria recovered.	
	Spleen.	Blood.
<i>days</i>		
6	<i>B. typhi exanthematici</i> Plotz.	None.
10	<i>Staphylococcus aureus</i> .	<i>Staphylococcus aureus</i> .
8	" "	" "
5	None.	<i>B. proteus</i> .

To sum up the results given in the tables, it may be stated that the typhus virus can be obtained during the period of incubation of the experimental disease free from admixture with any of the ordinary bacteria, while various bacteria are encountered during the febrile reaction, the variation in kind and the number becoming larger in proportion to the duration of the fever.

*Pathogenicity Experiments.*

It was noted that typhus guinea pigs in which Plotz' bacilli, anaerobic streptococci, aerobic diphtheroids, and Gram-positive diplobacilli were encountered, showed the same pathological picture as that found in the animals with the experimental disease in which no bacteria were present. Our attention was therefore directed to the probable non-pathogenic nature of these microorganisms.

We proceeded to put this idea to an experimental test. For this purpose four different cultures of the Plotz bacillus in the Smith-Noguchi medium were obtained from the blood of guinea pigs during the febrile reaction of the experimental disease. The bacterial content in 10 cc. of this medium after 7 days incubation was centrifuged, suspended in 4 cc. of saline solution, and inoculated intraperitoneally into four guinea pigs. No action was noted during a period of observation from 24 to 30 days. At the conclusion of this experiment the four guinea pigs were inoculated with the usual amount, namely 3 cc., of guinea pig blood containing typhus virus, two of the guinea pigs with the human strain of the Polish virus and two with the louse strain of the same virus. After an average period of incubation of 9 days all showed febrile reactions indicative of experimental typhus fever and the blood was found to be capable of transmitting the typical disease to other guinea pigs. Thus the cultures of *Bacillus typhi exanthematici* failed either to induce the experimental disease or to set up immunity to a subsequent injection of the typhus virus. The anaerobic streptococci, aerobic diphtheroids, and the Gram-positive diplobacilli tested in the same manner also failed.

## DISCUSSION AND SUMMARY.

The work reported in this paper relates to the bacteria which can be cultivated from the blood and spleen of guinea pigs at different stages of infection with the virus of typhus fever.

The studies show that during the period of incubation and before the onset of fever no ordinary bacteria appear in the cultures, while on the 1st day of the febrile reaction different bacteria were found in 6 of 26 guinea pigs cultured; on the 2nd day, in 10 of 16; on the 3rd day, in 3 of 4; and on the 4th day in cultures of all of the 4 guinea

pigs observed. The findings indicate that the virus of typhus fever is distinct from ordinary cultivable bacteria, and, as the disease set up by the virus progresses, the infected guinea pigs become subject to invasion by secondary or concurrent bacteria which thus induce a mixed infection.

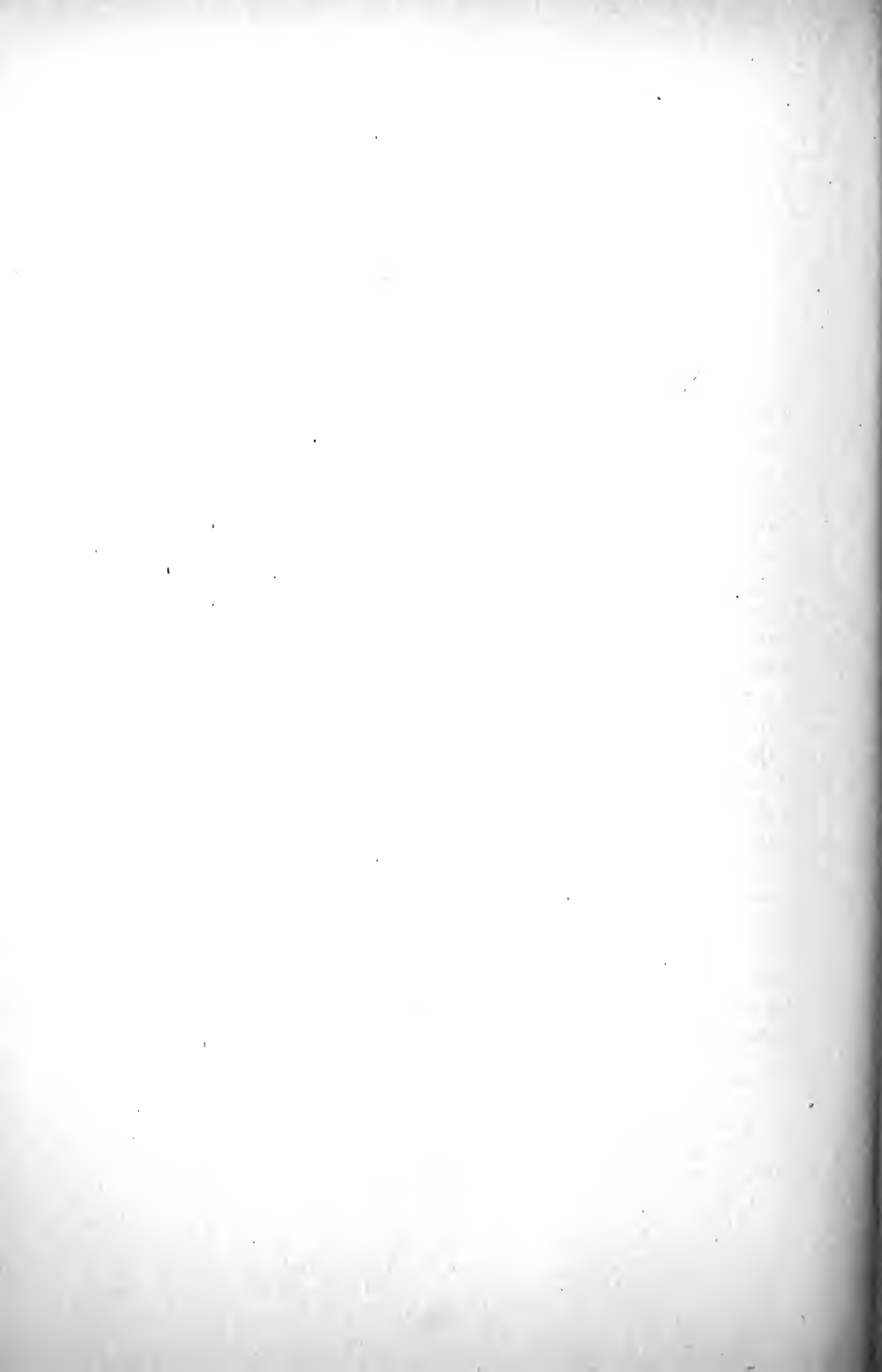
The bacteria which under the influence of the virus of typhus fever thus invade the body of the guinea pig are of several kinds, and vary not only among themselves, but also with the day of the fever on which the examination is made. Thus, on the 1st day of the fever Plotz' bacilli were recovered twice and anaerobic streptococci, *proteus* bacilli, aerobic diphtheroids, Gärtner type bacilli, and *Staphylococcus aureus* each once. On the 2nd day Plotz' bacilli were found four times, anaerobic streptococci three times, Gärtner type bacilli, aerobic diphtheroids, *Bacillus welchii*, aerobic Gram-positive diplobacilli, and *Staphylococcus aureus* each once. On the 3rd day Plotz' bacilli were recovered once, as were anaerobic streptococci and Gärtner type bacilli. On the 4th day *Staphylococcus aureus* was found twice and Plotz' bacilli and *Bacillus proteus* each once.

This variation in the kind of bacteria as well as the lack of predominance of one kind over another during the different stages of the febrile reaction in guinea pigs leads us to infer that they occur concurrently with the typhus virus. And since the more unusual of these organisms, the Plotz bacillus, the anaerobic streptococcus, the aerobic diphtheroid, and the diplobacillus are non-pathogenic for guinea pigs, while the more common bacteria such as the Gärtner type bacillus, Welch's bacillus, the *proteus* bacillus, and the staphylococci induce distinctive effects, and since all the bacteria could be suppressed without their reappearance in guinea pig passages of the virus containing them, we believe that they are independent and unrelated to the true virus of typhus fever.

#### CONCLUSION.

In the early stages of experimental typhus fever in guinea pigs, the typhus virus can be obtained wholly free from admixture with any of the ordinary bacteria.

The body of the guinea pig reacting to the virus of typhus fever is readily invaded by a variety of bacteria whose presence complicates the typhus infection, but which have no etiological relation to the specific disease, typhus fever.





## EXPERIMENTAL MUMPS MENINGITIS.

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Meningitis or meningoencephalitis is a complication of parotitis which occurs in children as well as in adults. The incidence varies greatly. The highest number of cases recorded is 16 among 653 mumps patients observed by de Massary, Tockmann, and Luce,<sup>1</sup> while none occurred among 1,059 soldiers seen by Brooks.<sup>2</sup> It may be said that while mumps meningitis is of rare occurrence, it appears with sufficient frequency to be kept in mind by everyone called upon to treat patients ill with parotitis. The nervous symptoms may precede or follow the parotid swelling. More often, however, symptoms of meningeal irritation develop during the height of the parotid lesion.

In work on experimental parotitis<sup>3</sup> in cats, it seemed interesting to attempt to produce meningitis by means of the virus which caused the characteristic lesions in the salivary glands in the inoculated animals. With four strains of active virus, intrathecal inoculations gave only negative results in ten cats. Finally, a virus was obtained from the mouth secretions of four children on the 2nd and 3rd days of an attack of typical parotitis.<sup>4</sup> The filtrate from the pooled mouth washings was inoculated into the parotid glands of two cats and into the subarachnoid space of two others. All the animals used in these experiments were young cats of medium size. The two which had been inoculated into the parotid gland developed typical signs of parotid swelling, leucocytosis, and fever. When killed on the 16th day, the parotid, submaxillary, and adjacent lymph glands were swol-

<sup>1</sup> de Massary, E., Tockmann, and Luce, *Bull. Acad. méd.*, 1917, lxxviii, 6.

<sup>2</sup> Brooks, H., *Med. Clin. North America*, 1918-19, ii, 492.

<sup>3</sup> Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 353; 1918, xxviii, 377.

<sup>4</sup> We are indebted to the courtesy of Dr. J. Lewengood, Attending Physician at the Hebrew Orphan Asylum, for this material.

len, congested, and moist. Microscopically, they showed the epithelial swelling, edema, and interstitial infiltration characteristic of human and experimental parotitis. A protocol of an intrathecal inoculation experiment follows, and is typical.

*Cat A.*—Mar. 10, 1921. Temperature 38.4°C. Subarachnoid occipito-atlantoid puncture withdrew clear fluid, containing no cells and giving a negative globulin test with the Noguchi method.<sup>5</sup> Cultures proved negative on ordinary media. 1 cc. of sterile mouth filtrate pooled from four cases of mumps, 1 and 2 days in duration, was injected.

Mar. 11. The cat is quiet and refuses food. There is internal strabismus of the left eye. The animal walks slowly, but there is no paralysis. No convulsions. Puncture of the cisterna magna withdrew 1.5 cc. of turbid fluid under greatly increased pressure. No bacteria were seen in spreads, and cultures did not grow. Globulin ++; there were 22,000 cells, of which 84 per cent were polymorphonuclears and 16 per cent mononuclear leucocytes.

Mar. 12. Temperature 40°C. Strabismus is more marked. The cat walks only when disturbed, and is irritable when handled. No convulsions. Cerebrospinal fluid is still turbid, containing 18,000 cells, of which 66 per cent are polymorphonuclears. Globulin ++. Cultures did not grow, aerobically or anaerobically.

Mar. 14. Temperature 40°C. The animal is prostrated and the strabismus is still present. Puncture of the cisterna magna withdrew fluid under increased pressure, but almost clear. The cells numbered 500. Globulin +. Cultures remained sterile.

Mar. 15. Temperature 39.9°C. Cerebrospinal fluid clear. The strabismus has disappeared. The cat is thin.

Mar. 16. Temperature 39°C. Though thin and quiet, the animal is apparently well.

Another cat injected into the subarachnoid space with this same strain of parotitis virus gave symptoms similar to those described above, and was killed with chloroform on the 3rd day. The pia mater over the cortex was found to be cloudy but no exudate was present. The vessels of the pia mater were deeply congested over all surfaces of the brain. The ventricles were not dilated. Anaerobic and aerobic cultures proved negative. Microscopic examination showed that all the vessels were filled with blood cells and edema of the pia was marked. There were only a few small groups of polymorphonuclear cells around some of the smaller vessels. The lesion of the pia mater is mild in character just as the lesion of the parotid is.

<sup>5</sup> Noguchi, H., *J. Am. Med. Assn.*, 1921, lxxvi, 632.

It is possible, then, by injecting sterile mouth filtrate from early mumps cases into the subarachnoid space of cats, to cause signs of meningeal irritation. These are: increase in the amount of cerebrospinal fluid with an abnormal content of cells and globulin, increased intrameningeal pressure, and consequent symptoms of irritability, ocular irregularity, prostration, and fever.

It remained to attempt to transmit the meningeal lesion from one cat to another. This was done with the cerebrospinal fluid from Cat A and from one other cat which had reacted characteristically. The following is a typical protocol of such a transmission experiment.

*Cat B.*—Mar. 21, 1921. Temperature 38.6°C. Occipito-atlantoid puncture withdrew clear fluid, containing 20 cells and reacting negatively to a test for globulin. Cultures did not grow. 0.5 cc. of slightly turbid, sterile, cerebrospinal fluid obtained from Cat C on the 3rd day after occipito-atlantoid inoculation with active mumps virus was injected into the cisterna magna.

Mar. 22. Temperature 40.3°C. The animal lies in the back of the cage and refuses food. No paralyses are apparent. The left pupil is more widely dilated than is the right. On puncture of the cistern, turbid cerebrospinal fluid escapes under high pressure. Only 0.5 cc. could be caught in a test-tube, and it contained an increased amount of globulin with 2,300 cells, of which 80 per cent were polymorphonuclear leucocytes. Cultures proved negative.

Mar. 23. Temperature 40°C. The animal is still quiet, the cerebrospinal fluid is less turbid, and globulin +. No bacteria present.

Mar. 24. Temperature 39.5°C. The eyes are normal. The cat walks slowly and the appetite is improved. Cerebrospinal fluid is clear and sterile.

Recovery would seem to be the rule in these cases. The only animal which died succumbed on the 2nd day because in puncturing the cisterna magna the needle entered the medulla.

Three controls were made. Sterile salt solution was injected into the cisterna magna of a cat and caused no symptoms other than a mild rise in temperature of 0.8°C. for 1 day; the cerebrospinal fluid withdrawn was clear. Another control was made by injecting into the cisterna 1 cc. from a sterile, fluid, anaerobic culture which had been inoculated 10 days before with sterile extract from the experimentally swollen parotid gland of a cat. The injection was followed by no symptoms. The temperature rose only 0.4°C. The cerebrospinal fluid of the cat remained clear, sterile, and without a demonstrable amount of globulin. The animal was not prostrated, nor did it lose weight. The third control was made with the filtrate of

saliva from a normal individual; the injected cat developed no symptoms.

The second control was for the purpose of noting the effect of the injection of a fluid rich in protein on the meninges, since the culture medium was human ascitic fluid.

The sterile filtrate from the mouth washings of patients suffering from an attack of parotitis of 1 or 2 days duration, when injected into the cisterna magna of young cats by occipito-atlantoid puncture, causes signs and symptoms of meningitis of 3 to 5 days duration, which end in recovery. The picture resembles that of mumps meningitis as it occurs in human beings, but differs from it in the high polymorphonuclear cell reaction as evidenced by the cerebrospinal fluid. Flexner and Amoss<sup>6</sup> showed that the injection of sterile horse serum into the subarachnoid space of monkeys caused an aseptic inflammation of mild degree, which reached its maximum in 24 hours. Polymorphonuclear cells predominated in the exudate. A polymorphonuclear reaction was also found by Ayer<sup>7</sup> in aseptic meningitis caused by the injection of normal rabbit serum into the meninges of cats; but this meningitis was of only 1 day's duration and accompanied by no general symptoms. However, the fact that in our experiments neither sterile salt solution, sterile ascitic fluid, nor sterile saliva from a normal person caused the meningeal reaction brought about by the injection of the filtrate containing an active mumps virus, would seem to argue that the reaction caused by the filtrate was due to some factor other than either albuminous fluid or salt solution.

#### SUMMARY.

It has been shown that an aseptic meningitis of 3 to 5 days duration and favorable prognosis can be induced in cats by intrathecal injection of sterile saliva filtrate from early cases of parotitis, and that such a meningitis can be transmitted to other cats by injecting the cerebrospinal fluid in the same manner. The cerebrospinal fluid does not, at any time, contain bacteria which grow with the ordinary culture methods.

<sup>6</sup> Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.

<sup>7</sup> Weed, L. H., Wegforth, P., Ayer, J. B., and Felton, L. D., A study of experimental meningitis. A series of papers from the Army Neuro-Surgical Laboratory, Monograph of The Rockefeller Institute for Medical Research, No. 12, New York, 1920, 36.

# AN OUTBREAK OF PNEUMONIA IN DAIRY COWS ATTRIBUTED TO *BACILLUS BOVISEPTICUS*.

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PLATES 44 TO 46.

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Bollinger<sup>1</sup> in 1878 described under the name *Wild- und Rinderseuche* an acute disease in deer, wild boars, and cattle. He was able to recognize two types, exanthematous and pectoral. In 1885, Kitt<sup>2</sup> isolated a bacterium (*B. bovissepticus*) from a disease of cattle similar to that described by Bollinger. Many outbreaks of what is termed hemorrhagic septicemia have been described in various species of bovines. That the disease existed in cattle in this country was recognized by Theobald Smith<sup>3</sup> in 1895. Nocard<sup>4</sup> a few years before described its occurrence and succeeded in isolating the bacillus from cattle shipped from America to France. Many have confirmed Bollinger's observation that the malady attacks practically all species of bovines. Wilson and Brimhall<sup>5</sup> during 1900 studied in Minnesota eleven outbreaks of disease in cattle caused by *B. bovissepticus*. The disease was of short duration, death ensuing within 24 hours after the onset. On autopsy, ecchymoses and larger hemorrhages were found in the subcutis, muscles, and internal organs. Pneumonia was not observed. Woolley<sup>6</sup> in the Philippines reports the pulmonary form in caribou. Mohler and Eichhorn<sup>7</sup> attribute losses among the buffaloes in the Yellowstone National Park to infection with *B. bovissepticus*. Magnusson<sup>8</sup> describes a very severe type of hemorrhagic septicemia in the reindeer of Lapland.

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<sup>1</sup> Bollinger, O., Ueber eine neue Wild- und Rinderseuche welche im Sommer 1878 in der Umgebung von München beobachtet wurde, Munich, 1878.

<sup>2</sup> Kitt, Th., *Sitzungsber. Ges. Morphol. u. Physiol.*, 1885, i, 140.

<sup>3</sup> Smith, T., *U. S. Dept. Agric., Bureau Animal Industry, 12th and 13th Ann. Rep.*, 1895-96, 119.

<sup>4</sup> Nocard, E., *Rec. méd. vét.*, 1891, viii, 424.

<sup>5</sup> Wilson, L. B., and Brimhall, S. D., Sixty cases of hemorrhagic septicemia in cattle due to *Bacillus bovissepticus*—A report of the State Board of Health of Minnesota, St. Paul, 1901.

<sup>6</sup> Woolley, P. G., *U. S. Dept. Interior, Bureau Gov. Lab., Philippine Islands, Bull.* 12, 1904.

<sup>7</sup> Mohler, J. R., and Eichhorn, A., *Am. Vet. Rev.*, 1912-13, xlii, 409.

<sup>8</sup> Magnusson, H., *Z. Infektionskrankh. Haustiere*, 1914, xv, 61.

In this country the disease commonly occurs in beef cattle which have been shipped to various parts of the country for fattening. The outbreak with which we are concerned occurred in a dairy herd. Throughout the literature we are unable to find detailed descriptions of outbreaks of pneumonia in cattle where it was possible to obtain a definite history of the herd in which the disease occurred. In this instance it has been possible to follow cases from early in the course of the disease to autopsy or recovery. In addition, several opportunities have been afforded for observations on sporadic cases which occurred after the main outbreak had subsided. We feel that the data accumulated warrant publication.

*Conditions before the Outbreak.*

The herd in which the disease occurred has been under observation by members of the staff of this Department since 1917. During this time a considerable number of autopsies of cows and calves have been made. Theobald Smith<sup>9</sup> noted that during 1917 cases of pneumonia occurred among the calves. One case was regarded as a primary infection with *Bacillus bovisepiticus*. During 1919 and 1920 he studied twelve cases of calf pneumonia. In nine instances *Bacillus actinoides* was found. It is stated that *Bacillus bovisepiticus* was associated with three or four of these nine cases. It will be shown in a later communication that the type of *Bacillus bovisepiticus* which was encountered during the epidemic to be described differs in many respects from the types which were associated with the pneumonia of the calves. The inference then is that the bipolar organism associated with the epidemic was not present in the herd but was introduced through the purchase of new cows.

With this herd the policy has been to purchase cows as circumstances dictate. Usually a number of animals are procured from various farmers. They are assembled and shipped by express. It is customary to segregate newly imported animals in separate barns. Such cows are milked and fed after the regular herd. The following importations were made during the later months of 1920: August 18, 24 cows from Pennsylvania; October 1, 12 cows from Pennsylvania;

<sup>9</sup> Smith, T., *J. Exp. Med.*, 1921, xxxiii, 441.

October 20, 39 cows from Michigan. Both lots from Pennsylvania came by express and were unloaded only at the final destination. All animals received from Michigan came by freight in stock and box cars as it was not possible to obtain express cars at that time. This lot of Michigan cows was loaded October 16. The following day they were unloaded at Mansfield, Ohio, and there fed and watered. The same procedure was gone through at Pittsburgh, Pennsylvania, on October 18. From Pittsburgh they were forwarded directly and reached their destination on October 20. These animals, the Pennsylvania cows, and a few others purchased from local farmers, together with a few native cows, were housed in the same barn.

#### *The Outbreak.*

One of the Michigan cows was sick when received. Symptoms of pneumonia were not noted during its illness. The animal remained unthrifty and finally became valueless for dairying purposes. It was slaughtered in April, 1921. The right cephalic lobe was shrunken and a gray-red color. It cut with difficulty. The interlobular connective tissue was increased. The lobules were compressed. The bronchi were greatly dilated and filled with pus.

On October 31, 1920, another of the Michigan cows was reported sick. It became worse, symptoms of respiratory disturbance developed, and the animal died during the night of November 2. Unfortunately, no autopsy was made. The man who disposed of the carcass reported a reddening of the lungs and their adherence to the chest wall. The next cow (No.531) sickened on November 3. During the next 2 weeks about twenty cases developed. It was possible to transport the first nine cases to an isolation unit at the Institute where facilities for careful study were at hand. Later two calves were also obtained from the same farm. The nine cows were the first in which the disease was recognized and are given in Table I in the order of their occurrence. One of the infections in the calves is added for completeness. Several other cases occurred in the herd but were not studied.

On the temperature charts it will be noted that certain animals were injected with vaccine and commercial hemorrhagic septicemia antiserum. The animals were not under the control of the Institute.

TABLE I.

Cow No.	Locality from which animal was obtained.	Date of introduction into herd.	Date first symptoms were noted.	Temperature.	Summary of characteristic symptoms.	Termination.
		1920	1920	°C.		
3809	Michigan.	Oct. 20	Oct. 31		Depression, cough, dyspnea, "grunting" on expiration.	Died Nov. 2, 1920.
531	"	" 20	Nov. 3	39.0	Superficial breathing, gummy discharge from eyes, inappetence and constipation, albuminuria.	Recovered.
533	Pennsylvania.	" 1	" 6	41.1	Depression, cough, dyspnea, dullness under areas of percussion, bronchial breathing, inappetence and constipation.	Killed Nov. 13. Diffuse pneumonia.
530	"	" 1	" 7	41.5	Cough, dyspnea, dullness on percussion, bronchial breathing, inappetence and constipation.	Killed Nov. 11. Diffuse pneumonia.
532	"	" 1	" 8	41.1	Cough, rapid respiration, dyspnea, emaciation, inappetence and constipation.	Recovered.
535	Local.	Nov. 3	" 10	41.8	Cough, rapid respiration, nasal discharge, inappetence and constipation, albuminuria.	"
534	Michigan.	Oct. 20	" 10	41.7	During observation animal revealed no characteristic symptoms.	"
538	Native.		" 11	40.8	Cough, rapid respiration, dyspnea, inappetence and constipation.	"
536	"		" 11	41.1	Dyspnea, dullness on percussion, bronchial breathing, nasal discharge, inappetence and constipation.	Killed Dec. 12. Multiple abscesses of lungs; unresolved pneumonia.

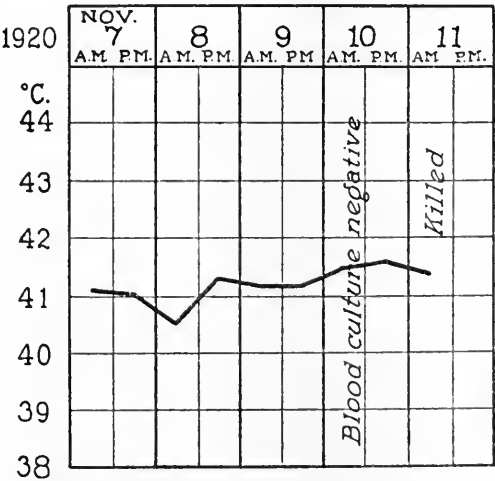


537	Pennsylvania.	Oct. 1	Nov. 13	40.2	Chills, dyspnea, dullness on percussion, bronchial breathing, congestion of conjunctiva, inappetence and constipation, albuminuria.	Died Nov. 18, Diffuse pneumonia.
Calf 562	Native.	Born Oct. 4.	<sup>1921</sup> Jan. 19	40.5	Chills, cough, dyspnea, rapid respiration, dullness, bronchial breathing, constipation followed by diarrhea, albuminuria.	Died Jan. 25, 1921. Diffuse pneumonia.

The injections were made by the owner's representative. Our limited observation concerning their value does not warrant any discussion.

The table indicates that the first cow to come down with pneumonia was one of those obtained from Michigan. The second case also occurred among this lot. The disease next attacked the Pennsylvania cows and further spread to the native stock. The mortality was highest in the Pennsylvania cows. The native stock suffered severely.

The records of several typical cases follow. The temperature charts are appended (Text-figs. 1 to 5).



TEXT-FIG. 1. Temperature chart of Cow 530.

*Cow 530.*—A grade Holstein cow purchased in Pennsylvania on Oct. 18, 1920. On Nov. 4, she gave birth to a normal calf. Nov. 7. The animal was reported sick. The temperature was 41.5°C. The next day the highest temperature recorded was 41.4°C. at 7 p.m. The respiration rate was accelerated. The milk flow was diminished, the appetite poor. The cow was constipated.

On Nov. 9, the cow was transferred from a neighboring farm to an isolation unit at the Institute. Weakness was apparent, the gait was unsteady. The respirations were labored, dyspnea was pronounced. On expiration the dyspnea was marked and accompanied by a distinct grunting sound. At times the animal breathed through the mouth. There was a thin white mucous discharge from both nostrils.

Nov. 10. Weakness was more apparent. The cow was restless. The head was extended, the nostrils dilated, and the mouth held open. Sudden movements of the limbs caused discomfort. The animal refused food and water. Milk secretion was suppressed. There were constipation and atony of the rumen.

The tail and buttocks were soiled with blood-stained, fetid feces. The skin was dry and hot. The conjunctiva was congested. Moderate pressure applied to the pharynx produced considerable discomfort. The cough was short, frequent, and painful. Temperature 41.6°C. The pulse was rapid and irregular (98 per minute). The respiration rate was 40. Respirations were short and superficial. Percussion of the thorax caused pain, there was dullness beneath most of the area of percussion. On auscultation the vesicular murmur was exaggerated and coarse and there was bronchial breathing.

The results of the urine examination were as follows: color, clear amber; specific gravity, 1.022; reaction, alkaline; albumin, present (Esbach 0.25 per cent); sugar, absent; bile, present; blood, absent.

Under antiseptic precautions, 15 cc. of blood were drawn from the left jugular vein into a flask containing 50 cc. of sterile bouillon. The mixture remained sterile after 7 days incubation.

On Nov. 11, the cow was killed and autopsied at once. The following autopsy notes were made.

The carcass is that of a well nourished adult Holstein cow.

*Heart*.—There is a large amount of fat about the pericardium. The pericardium appeared normal. On the fat overlying the right auricle there are three irregular patches of gelatinous hemorrhagic exudate. The heart muscle appears normal.

*Lungs*.—The process is that of an extensive pneumonia involving all lobes. The pneumonia is more pronounced on the ventral aspect. The external anterior third of the first half of the cephalic lobe of the right lung (ventral aspect) is of a reddish gray color, firm, and liver-like. The pneumonic process on the dorsal surface is confined to a dark red band, 3 to 4 cm. wide, extending from the anterior to the posterior border. The second half of the cephalic lobe is not appreciably involved. The ventral lobe is solidified. The pleura over the external two-thirds is covered with a yellow, felt-like fibrinous exudate, 2 to 6 mm. in thickness (Fig. 1). It may be peeled off with gentle traction, leaving the thickened pleura with adhering tufts of exudate. The underlying lung is mottled dark red to reddish gray. The lobules stand out clearly due to a thickening of the interlobular septum and the filling of the lymph vessels with a serous exudate (Fig. 2.) In many instances coagulation of the exudate has occurred. This material may be "shelled out" from some of the larger lymph spaces. The lobules are solid. Their color varies from deep red to reddish gray. Tiny grayish white patches are observed scattered throughout many of them. The central bronchi are often occluded with a plug of coagulated exudate. From the cut surfaces a yellow, turbid, serous fluid exudes.

On the ventral surface of the right caudal lobe the anterior half is involved. The involved portion varies from reddish gray to gray in color. It is solid and presents the same gross characters as those described under the right ventral lobe. The portion of complete hepatization is succeeded by a band in which the consolidation is irregular. On section, some of the lobules are deep red and solid, others are pink and contain air. From the cut surfaces a clear serous fluid flows.

On the dorsal surface of this lobe the process is similar but it is confined to the anterior fourth. The azygos lobe is consolidated. It is mottled reddish gray. The pleura is covered with a yellowish white, fibrinous exudate.

In the left lung the process is confined to about the same areas. There is a reddish gray consolidation of the dependent portion of the cephalic lobe which is not as large on the dorsal aspect. A complete solidification of the lower aspect of the ventral lobe is observed. A small portion of the dorsal surface still shows air-containing tissue. About half of the caudal lobe (ventral aspect) is involved, but barely one-third of the dorsal surface is affected.

The pleural cavity contains a considerable amount of clear serous fluid which coagulates on standing. The parietal pleura, especially that in contact with the ventral and azygos lobes, is covered with a thick, felt-like exudate which in certain places has adhered to the lung pleura.

There is a patch-like congestion of the mucosa of the trachea. The mucosa of the bronchi is congested. Adhering here and there to the mucous membrane are masses of viscid mucus-like material.

The bronchial and mediastinal lymph glands are edematous. They are not enlarged or discolored.

*Liver.*—The organ is not enlarged. The color is light brownish red. The gall bladder is distended with blackish green, gelatinous bile.

*Spleen.*—Not enlarged, measures 3.8 by 11.5 cm. The vessels of the capsule are injected. Here and there are round, slightly raised hemorrhages 2 to 3 mm. in diameter. On section the capsule is not thickened. The pulp is normal in color. The Malpighian corpuscles are not enlarged.

*Kidneys.*—They are well surrounded by fat. Their color is reddish gray. No hemorrhages are visible on the surface. The capsule is not thickened and peels off readily. On section the vessels of the cortex are injected. When scraped the epithelium is dry and appears granular. The medulla is dark red and glistening. It is clearly demarcated from the cortex.

*Gastrointestinal Tract.*—Appears normal.

Pieces of the various organs were fixed in Zenker's fluid for microscopic study. The following changes were observed in the stained sections. The pleura is covered by a dense mass of fibrin. The pleural lymph spaces are filled with fibrin. Both red cells and leucocytes are enmeshed in the exudate. There is an increase in the endothelial elements. The exudate becomes more cellular as the lung is neared, so that immediately adjacent to the lung densely packed masses of leucocytes are found.

Two distinct stages of pneumonia can frequently be seen in the same section (Fig. 4). The alveoli in one lobule may be filled with fibrin and the alveolar capillaries packed with masses of red cells (Fig. 6). Very few cells have invaded the air spaces in such portions. In other lobules the lesion is that of extensive hemorrhage, the air spaces are largely filled with red cells. The blood vessels are engorged and not infrequently plugged with fibrin. The bronchi and bronchioles

contain fibrin and red cells. Such lobules are separated from an older type lesion by the thickened interlobular septum (Fig. 4). The septum where it leaves the pleura is edematous and at times shows proliferation of connective tissue elements. The interlobular tissue is edematous and swollen with a fibrinous exudate. The lymph channels and blood vessels are dilated and plugged with fibrin. In the pneumonia of longer standing (Fig. 7), the interlobular congestion is more moderate. The air spaces are filled with degenerated leucocytes and desquamated alveolar cells. Considerable numbers of tiny rod-shaped organisms are found in the exudate within the alveoli (Fig. 8). The epithelium of the bronchi reveals degenerative changes. The lumen is usually filled with leucocytes and bacteria.

Sections from a number of regions were examined. Various stages of pneumonia were observed. In certain sections the process is largely that of hyperemia. Other lobules show extensive hemorrhages. In others the alveolar exudate is largely fibrinous or fibrinopurulent or purulent.

Sections of the liver show extensive fatty changes in the liver cells. The nuclei are large and vacuolated, in places they are granular.

The capsular vessels of the spleen are injected and the pulp is congested.

The vessels of the kidneys are congested. There are slight degenerative changes in the epithelium of the cortex.

Microscopic examination of one of the mediastinal lymph glands reveals a congestion of the vessels of the cortex with an occasional area of fibrinous exudate about the vessels. The medullary portion is edematous. Some of the vessels contain fibrin, others are filled with polymorphonuclear leucocytes. Polymorphonuclears have invaded the lymph sinuses.

Inoculations from all involved portions of the lung and pleural fluid were made in various media. Both the platinum loop and pieces of tissue were employed. A platinum loop when brushed over a freshly cut surface and streaked over several agar slants in succession usually gave well isolated colonies in the third or fourth tube after 24 hours incubation. Pure cultures of *B. bovissepticus* developed in all tubes. The tubes inoculated with the pleural fluid contained the organism in pure culture.

Pieces of spleen and kidney introduced into tubes of media remained sterile after prolonged incubation. Mention has been made that a blood culture taken 24 hours before autopsy remained sterile.

In films from the lung stained with methylene blue or dilute carbolfuchsin, *B. bovissepticus* could be demonstrated in large numbers. Very few of the organisms revealed the characteristic bipolar staining. In the sections large numbers of bacteria are present in the alveolar (Fig. 8) and bronchial exudate.

*Cow 537.*—Grade Holstein purchased in Pennsylvania Oct. 1, 1920. Normal parturition occurred on Oct. 30, 1920.

On Nov. 13, the cow was reported sick. The temperature at 9 a.m. was 40.2°C. and at 9 p.m. 41.8°C. The respiration rate was 48 per minute. The respirations were abdominal in character. They were superficial and rapid. The animal was constipated.



yellow in color and turbid. The specific gravity was 1.026; reaction, alkaline; albumin, present (Esbach 0.1 per cent); bile, present; blood and sugar, absent.

This cow died Nov. 18 between 2 and 3 a.m. The following postmortem findings were noted.

*Heart*.—Normal in size. The pericardium is covered with a thick, yellowish, felt-like, fibrinous exudate. There is considerable blood-stained turbid pericardial fluid. Scattered over the heart are many irregular hemorrhages. They are especially numerous over the auricles. On section, the cardiac muscle appears dry.

*Right Lung*.—The ventral aspect of the cephalic, ventral, azygos, and anterior third of the caudal lobes varies in color from bright red to reddish gray. The consistency is firm. The consolidation on the dorsal aspect is not quite so extensive. A small portion of the ventral lobe (dorsal surface) is still air-containing. The area of complete solidification in the caudal lobe occupies the cephalic third of the ventral aspect. It is succeeded by a mottled portion containing both hepatized and air-containing lung. There are patches of hepatization extending well down to the caudal border. On section, the pleura is thickened. The interlobular tissue is 3 to 5 mm. in thickness, due to the presence of a coagulated exudate which fills the lymph channels (Fig. 3). The lobules are distinct. Their color varies from dark red to reddish gray. Scattered throughout the dark red lobules are tiny, irregular, gray areas. These foci in other lobules have become confluent and give the whole a reddish gray appearance. The cut surfaces of the red portions are moist and exude a serous fluid. The gray areas are dry and granular.

*Left Lung*.—The anterior half of the cephalic lobe is dark red and firm. It is clearly demarcated from the surrounding emphysematous portion, where the interlobular tissue is distended with air. The interlobular emphysema extends as a narrow band backward through the ventral lobe and widens out in the caudal lobe. The ventral lobe is consolidated except for the narrow strip along the median line (dorsal aspect). The anterior third of the caudal lobe is reddish gray and firm. This portion is sharply defined from the surrounding deep red consolidation. The pink air-containing lung is clearly demarcated from the solid area.

*Trachea*.—The mucosa is reddened in patches. The submucous capillaries are injected. There is a moderate amount of blood-stained, viscid exudate mixed with air. The larger bronchi reveal much the same condition. The principal bronchus of the left caudal lobe is completely occluded with clotted blood.

The bronchial and mediastinal lymph glands are enlarged and congested. On section, the cortex is reddish gray, the medulla grayish white and mottled with irregular hemorrhages. The pleural cavity contains between 2,200 and 2,300 cc. of a slightly blood-stained serous fluid. Suspended in the fluid are feathery particles of fibrin. The endothelium lining the ventral floor of the cavity is sprinkled with irregular hemorrhages and here and there coagulated tufts of exudate.

*Liver*.—Reddish yellow in color. The gall bladder contains a gelatinous, dark green bile.

*Spleen.*—The spleen is not enlarged. A few small hemorrhages are present in the capsule. The pulp is apparently normal.

*Kidneys.*—Both are of a deep reddish gray color. The capsule is easily stripped off. Scattered over the surface are tiny round areas of reddening, surrounded by a narrow gray zone. On section, the cortex is congested and appears granular. The line of demarcation between the cortex and medulla is indistinct. The medulla is highly congested.

*Gastrointestinal Tract.*—The only lesion exists in the small intestine. The serous surface is reddened, apparently due to a congestion of the small capillaries of the subserosa.

Fixed and stained sections from the lungs show much the same type of pneumonia as in the preceding case. The pleura and interlobular tissue contain dense masses of fibrin (Fig. 5). Certain lobes reveal the fibrinous pneumonia, in others the process is fibrinopurulent, or purulent. Enormous numbers of *B. bovissepticus* can be seen within the alveolar and bronchial exudate.

Much the same lesions of the liver are observed in this case as in Cow 530. The kidneys, however, show more marked changes. There is degeneration of the cells of the glomeruli and those of the tubular epithelium. Desquamation of the latter is not infrequent. Severe congestion of the larger vessels is frequent.

Films made from consolidated portions of the lung when stained reveal enormous numbers of short rods. A few stain only at the poles. About many of the organisms are clear areas which suggest capsules.

Inoculations into various media from portions of the lungs and the pleural fluid all developed *B. bovissepticus*. Other organisms could not be demonstrated.

We have previously stated that for a time the disease existed only in adult cows. There had, however, existed among the calves certain types of pneumonia. Theobald Smith has shown that *Bacillus actinoides*, an organism which can be grown only under special conditions, is really responsible for certain pneumonias in calves. *Bacillus bovissepticus* had been isolated by him from some of these cases. That such strains of *Bacillus bovissepticus* are different from the bacterium associated with this epidemic will be shown in a later communication.

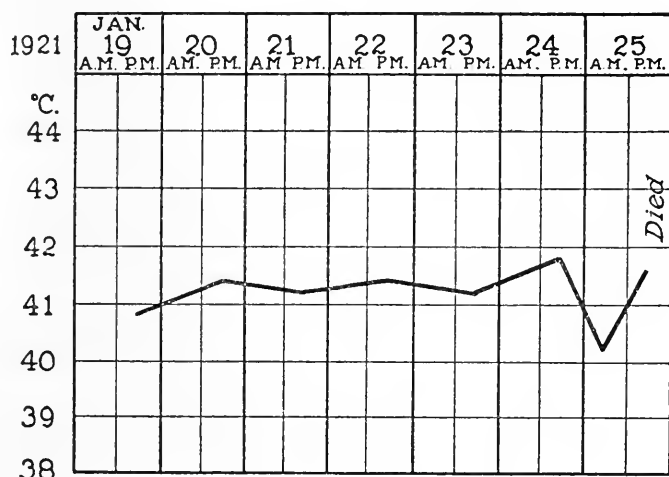
On December 7, 1920, a calf (No. 550) was reported sick. It was killed on December 17. Both *Bacillus actinoides* and a type of *Bacillus bovissepticus* similar to that found in the cows were isolated from the lungs. The colonies of *Bacillus actinoides* greatly predominated in all tubes. Nearly 1 month later what may be regarded as a primary infection with *Bacillus bovissepticus* occurred in Calf 562. The clinical and pathological records follow.



*Calf 562.*—Heifer calf, born Oct. 4, 1920.

On Jan. 19, 1921, the animal had a chill. The respirations were rapid. There was a discharge from both nostrils. The temperature was 40.5°C. Jan. 20. The temperature was 41.2°C., the respiration rate 100. The appetite was good. There was slight constipation. On auscultation the vesicular murmur was greatly increased. Râles were not heard. On Jan. 21, the temperature and respirations were the same. The calf coughed frequently. On Jan. 23, the temperature and respirations were about the same. Diarrhea had set in.

Jan. 25. Pulse 80, respiration 60, temperature 41.5°C. There was marked weakness. The respirations were shallow and accompanied on expiration with a distinct "grunt." On auscultation there was an absence of vesicular murmur in the cephalic lobes. There was a loud bronchial murmur especially over the caudal lobes (both lungs). Pressure over the lungs produced pain. The animal died



TEXT-FIG. 3. Temperature chart of Calf 562.

between 5.30 and 7.30 p.m. It was removed to the refrigerator over night and autopsied on Jan. 26. The following autopsy notes were made.

On removing the skin from the neck, the subcutis and fascia overlying the muscles are edematous. The small veins are dilated and many have ruptured, producing hemorrhages.

*Heart.*—Punctiform hemorrhages under the epicardium. The left auricle is distended with a large dark red clot. The ventricles also contain clots. There is a moderate hemorrhagic infiltration into the auriculoventricular valves.

*Lungs.*—There is nearly complete consolidation of the anterior lobes (cephalic and ventral) of both lungs. The azygos lobe is completely hepatized. The more dependent portions are reddish gray in color, the dorsal aspect is a darker red. The interlobular markings are distinct but are not appreciably thickened. On section these lobes are liver-like. The lighter colored portions reveal throughout the lobules tiny indistinct gray areas. These gray patches are larger about the bronchi.

In the dark red portions the gray areas are very tiny or absent. Blood oozes from the cut surface. Some of the smaller bronchi are occluded with purulent exudate. There is an extensive hepatization of both caudal lobes. The pneumonia occupies the anterior four-fifths of the right caudal and the anterior two-thirds of the left caudal lobe. It is less extensive on the dorsal aspect. The anterior portion varies from grayish red to dark red in color. This is succeeded by mottled areas of dark red consolidation and pink air-containing lung.

The pleura is not thickened and the pleural cavity does not contain an excess of fluid.

*Trachea and Large Bronchi.*—Scattered over the serosa of the trachea are round hemorrhages varying from very tiny points to extravasations 2 to 3 mm. in diameter. The mucosa is deep red. There are patches of mucopurulent exudate containing blood clots adhering to the mucosa. The same condition exists in the larger bronchi. Many of the smaller air tubes contain plugs of exudate and clotted blood.

The mediastinal and bronchial lymph glands are edematous. Tiny irregular hemorrhages are present throughout their substance.

The liver shows evidence of cloudy swelling. The spleen is normal in size. The kidneys are enlarged and of a deep red color. On section, blood oozes from the cut surfaces. The cortex is gray with deep red striations. Between the cortex and medulla is a deep red zone. The medulla is congested.

*Gastrointestinal Tract.*—The abomasum contains a few curds. The mucosa is deeply reddened. The small intestine contains much turbid fluid. The mucosa is deeply congested. The congestion is in the form of points, lines, and patches of varying size. The mucosa of the cecum and rectum is uniformly congested. Examination of a sample of straw-colored, turbid urine obtained from the bladder at autopsy revealed the following: specific gravity, 1.012; reaction, acid; albumin, present (Esbach 0.1 per cent); sugar, absent.

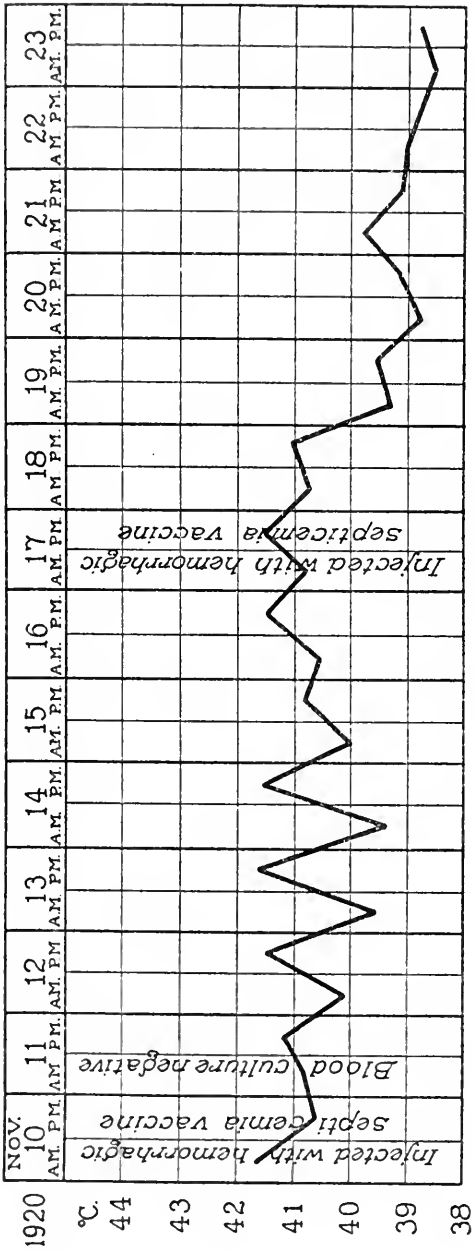
Examination of stained films from the involved lung shows characteristic bipolar bacilli in moderate numbers in all preparations. Pure cultures of *B. bovis septicus* developed from all tubes inoculated with bits of tissue or loopfuls of material obtained from cut surfaces.

Microscopic examination of fixed and stained sections reveals in general a pneumonia in which the air spaces are largely filled with polymorphonuclear leucocytes and desquamated alveolar cells. The interalveolar capillaries are congested. The bronchi are filled with leucocytes. In some the epithelium is intact. In others it is degenerated and often desquamated. The pleura is not thickened. There is a moderate edema of the interlobular septum. Scattered throughout some of the lobules are circular deep-blue-staining areas. They are composed of large numbers of intensely stained, degenerated leucocytes. Here the bacteria are present in large numbers.

In sections of the more recent pneumonia in the caudal lobes the process resembles more nearly that described for Cows 530 and 537. Here the pleura is thickened with masses of fibrin. The interlobular septa are also thickened, due to

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TEXT-FIG. 5. Temperature chart of Cow 535.

the dilatation and plugging of the blood and lymph spaces with fibrin. The peribronchial tissue is edematous. The surrounding blood vessels are dilated and plugged with fibrin. The interalveolar capillaries are highly congested. Hemorrhages into the air spaces have occurred. In certain lobules the extravasations of blood have obliterated the whole lobular structure.

The lesions in the kidney are much more severe in this case. The glomerular epithelium shows degenerative changes. Throughout the section congestion is severe. The tubular epithelium is degenerated. The remains of the cells appear as pink-staining, finely granular material often without definite nuclear structure.

*Cow 533.*—This cow was also killed after having been under observation for 10 days. Symptoms similar to those described under Cows 530 and 537 were noted. Autopsy revealed the same type of diffuse pneumonia. A blood culture taken late in the course of the disease remained sterile. *B. boviseplicus* was isolated in pure culture from various portions of the involved lung.

*Cow 536.*—This cow presented symptoms of an acute pneumonia similar to those described. The temperature chart, however, shows that the temperature remained above the normal for a considerable period. It finally reached normal and stayed so until the animal was slaughtered. The lungs were severely affected. The anterior lobes of both lungs were shrunk. They varied from gray to reddish gray in color. Scattered throughout these lobes were grayish white abscesses. On section the involved lobes cut with difficulty. The interlobular connective tissue was increased. The lobules were compressed and made up of distended bronchi filled with pus. The little remaining alveolar tissue was gray in color and dry in consistency. In many instances the lobules were entirely composed of thick walled connective tissue abscesses. Such abscesses often replaced a number of lobules. The anterior lobes adhered to each other and to the chest wall. A nail was found embedded in a dense mass of connective tissue in the anterior ventral tip of the left ventral lobe. The azygos lobe appeared normal. The trachea and large bronchi contained pus.

Many cultures were made from various portions of the lung. *B. pyogenes* was present in every tube. An unidentified micrococcus and anaerobes also developed in certain tubes. *B. boviseplicus* was not recovered from this case.

*Cow 535.*—The temperature record of this cow is appended. This animal suffered from what appeared to be a severe infection. Characteristic symptoms of pneumonia were observed. Albumin and bile were found in the urine. A blood culture taken 2 days after the first symptoms were noted remained sterile. The prognosis was grave but the temperature began to fall at the end of the 8th day. On the 9th day it fell from 41° to 39.3°C. From this day recovery was rapid.

#### DISCUSSION.

The outbreak is noteworthy in several respects. The origin of the infection appears at first glance more or less obscure. However,

evidence points toward the introduction of the disease with the Michigan cows. These animals were the last animals introduced into the herd. They had been fed and watered at two shipping points where the disease may have been contracted. One cow was sick on arrival, but pneumonia was not detected during life. This cow was killed 6 months later and examination of the lungs revealed a complete fibrosis with purulent bronchiectasis of the right cephalic lobe. Probably this cow gave rise to the epidemic. Another cow of this lot died 11 days after arrival. The clinical symptoms suggested pneumonia. No autopsy was made, but the man who cut up the carcass noted a reddening of the lungs and their adherence to the chest wall. The brief history which we were able to obtain in this instance indicated an illness of 2 days duration. From these animals the disease probably spread to the Pennsylvania and native cows. The greatest mortality occurred in those from Pennsylvania.

An outbreak with a similar history is recorded by Woolley and Jobling.<sup>10</sup> A shipment of caribou was received at Manila, P. I., from Shanghai on May 28, 1903. The animals were kept aboard ship until June 1 when they were sent to an estate. On June 2, two animals were sick. One died and the other was killed. The average time required to ship cattle from Manila to Shanghai is 5 days. The first cases occurred about 10 days after embarkation.

There is evidence that more cases occurred than the records indicate, especially when Cow 536 is considered. Since this outbreak subsided, two animals which were slaughtered revealed multiple abscesses of the lungs and purulent bronchitis. As far as could be learned, neither of these had ever shown recognizable symptoms. It is possible that a number of cows suffered from a very mild infection which passed unnoticed. During March and April, 1921, a few sporadic cases have occurred from which it has been possible to cultivate, in one instance, an organism identical with those met with in Cows 530, 533, and 537. These sporadic infections may continue to appear for some time.

The appearance of the disease in the calves is difficult to explain. They are kept at a considerable distance from the cows and have

<sup>10</sup> Woolley, P. G., and Jobling, J. W., *U. S. Dept. Interior, Bureau Gov. Lab., Philippine Islands, Bull. 9*, 1904.

separate attendants. Inquiry brought out the fact that calves born of the Michigan cows had been placed in the calf barns. We have no record of the epidemic type of *bovissepticus* pneumonia among the calves until December 7, 48 days after the purchase of the cattle from Michigan. Some of the Michigan calves, however, may have suffered from a very mild disease which may have been overlooked. It is possible that one may have been a virus carrier. It is of interest to note that the first calf (No. 550) from which the epidemic organism was isolated suffered from a primary infection with *Bacillus actinoides*. The other calf developed a pure *bovissepticus* infection. Both were of native stock. More recently another calf died after a short illness. *Bacillus bovissepticus* was obtained from the internal organs.

Another striking feature is the low virulence of the isolated bacteria, although the organism was capable under certain conditions of causing serious disturbances once it gained a foothold within the lung. The many apparent recoveries and the mild type of disease manifested in certain animals are also indicative of a low virulence. This fact is further borne out by inoculation experiments with rabbits and calves. In one experiment a calf was injected subcutaneously with 2.5 cc. of pleural exudate from Cow 537. Cultures made from this exudate revealed over 25,000 colonies per cc. The calf suffered no apparent ill effects. Another calf (No. 293B) was injected intratracheally with 10 cc. of a 24 hour bouillon culture of the organism isolated from Cow 530. The calf remained well. Similar negative results from intratracheal injections of cultures have been reported by others. Magnusson injected a sheep intratracheally with reindeer pasteurella without result. Pfeiler<sup>11</sup> states that infectious material obtained from cases of buffalo disease injected into the pectoral cavity or lungs of calves produced disease. On the other hand, when the material was introduced into the nasal passages or trachea the calves remained well.

The resemblance of the lung lesions in certain outbreaks of bovine septicæmia hæmorrhagica to those of contagious pleuropneumonia has been commented on by many. To rule out contagious pleuropneumonia at the start, a calf was injected subcutaneously with a

<sup>11</sup> Pfeiler, W., in Friedberger, E., and Pfeiffer, R., Lehrbuch der Mikrobiologie, Jena, 1919, ii, 879.



filtrate prepared by grinding 5 gm. of lungs with sterile sand. The ground material was suspended in 100 cc. of 0.85 per cent salt solution and shaken vigorously. The suspension was centrifuged at moderate speed. The supernatant liquid was passed through a Berkefeld V bougie. This calf was under observation for 30 days, during which time a local reaction did not develop. The calf's temperature remained well within the normal limits. Mention has been made of an experiment in which a calf was injected with pleural exudate. Only negative results were obtained.

Another striking feature is the localization of the virus within the lungs and chest cavity. Blood cultures taken from seven cases during the height of the disease remained sterile. *Bacillus bovisepiticus* failed to develop in tubes inoculated with bits of spleen and kidney from the cow that died and those that were killed.

#### SUMMARY.

An outbreak of pneumonia in dairy cows attributed to *Bacillus bovisepiticus* is described. About twenty cows and two calves were affected. The first cases were noted in cows purchased in Michigan. The disease next appeared in another lot of cows from Pennsylvania and subsequently attacked native stock. Of the ten cases which came under our observation, five apparently recovered, two died, and two severely affected cows were killed. The other case developed multiple abscesses of the lung and was finally killed.

The more characteristic symptoms observed were high temperature, rapid respiration, dyspnea, cough, dullness on percussion, bronchial breathing, and albuminuria.

The pneumonia was diffuse but first affected the cephalic and more dependent lobes. The process varied from hyperemia and hemorrhage to exudation of fibrin and leucocytes within the air spaces. The plugging of the interlobular lymph channels and blood vessels with fibrin was frequent.

*Bacillus bovisepiticus* was isolated in pure cultures from all involved portions of lung at autopsy. It was not found in blood cultures during the height of the disease, nor could it be obtained from the spleen and kidney after death.

## EXPLANATION OF PLATES.

## PLATE 44.

FIG. 1. Lung of Cow 530. Showing the thickening of the pleura and interlobular septum. The lobules are consolidated. In the center the lobules are gray, at the periphery the color is deep red.

FIG. 2. Lung of Cow 530. Interlobular septa are thickened with fibrin. The consolidation of all lobules is complete. A few are red in color, the others gray.

FIG. 3. Lung of Cow 537. Moderate pleuritis with large plugs of fibrin in the interlobular lymph spaces. Hepatization varies from red to gray.

## PLATE 45.

FIG. 4. Lung of Cow 530. The interlobular septum, greatly increased by a fibrinous exudate, separates two lobules. The alveoli in the upper lobule are filled with fibrin. Below the septum the air spaces also contain fibrin at the border of the lobule, but toward the center they have been invaded by leucocytes.  $\times 17$ .

FIG. 5. Lung of Cow 537. Lesions about the same as those in Fig. 4. Note fibrinous plugs in the vessels and lymph spaces of the interlobular septum. Above, the pneumonia is largely fibrinous; below, it is cellular.  $\times 17$ .

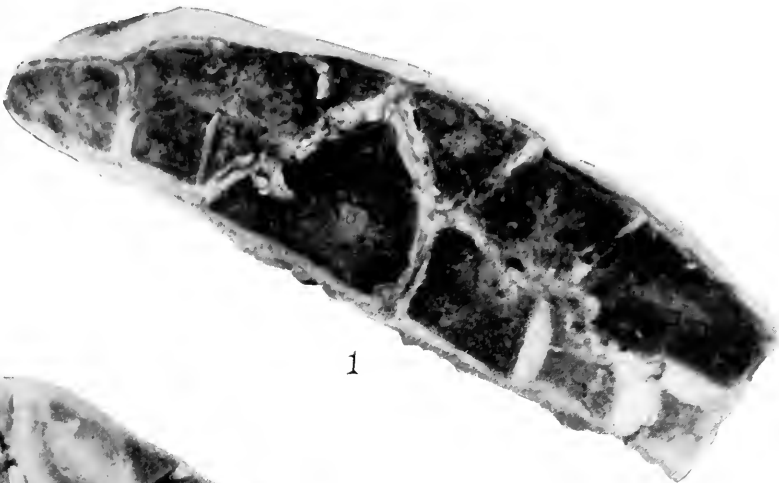
## PLATE 46.

FIG. 6. Lung of Cow 530. Reveals at the right the fibrinous exudate within the septum. The interalveolar capillaries are filled with red cells and the alveoli contain fibrin. A few leucocytes are observed within some of the air spaces.  $\times 110$ .

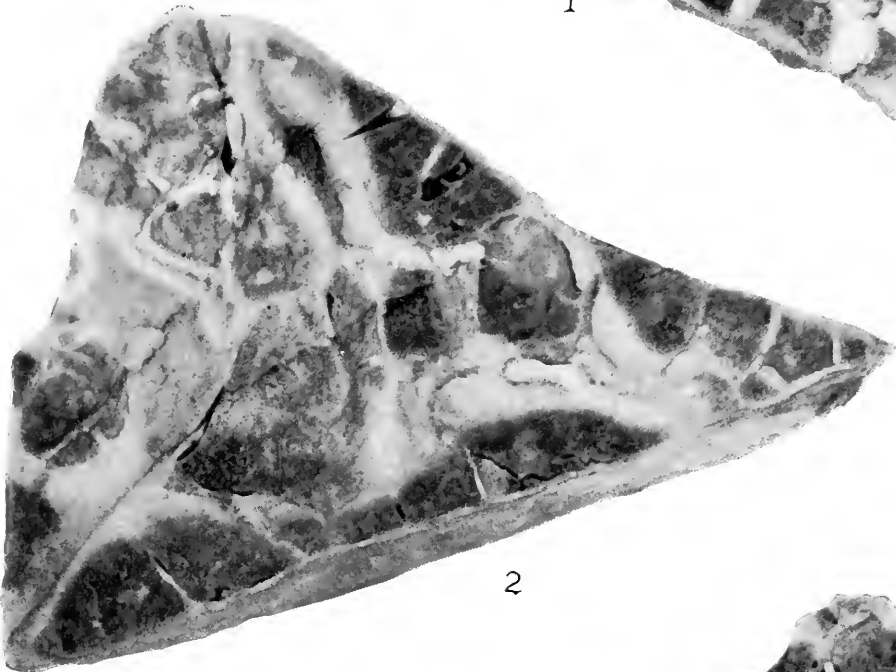
FIG. 7. Lung of Cow 530. A portion of a lobule adjacent to the one in the preceding photograph. The fibrinous exudate within the interlobular septum appears above. Below, the alveoli are packed with leucocytes. Some contain both leucocytes and fibrin. The interalveolar congestion is not marked.  $\times 110$ .

FIG. 8. Lung of Cow 530. *B. bovisepiticus* in considerable numbers is shown in the space between the alveolar wall and the cellular exudate. A dense mass of the organism is visible within the exudate.  $\times 1,000$ .

360'



1



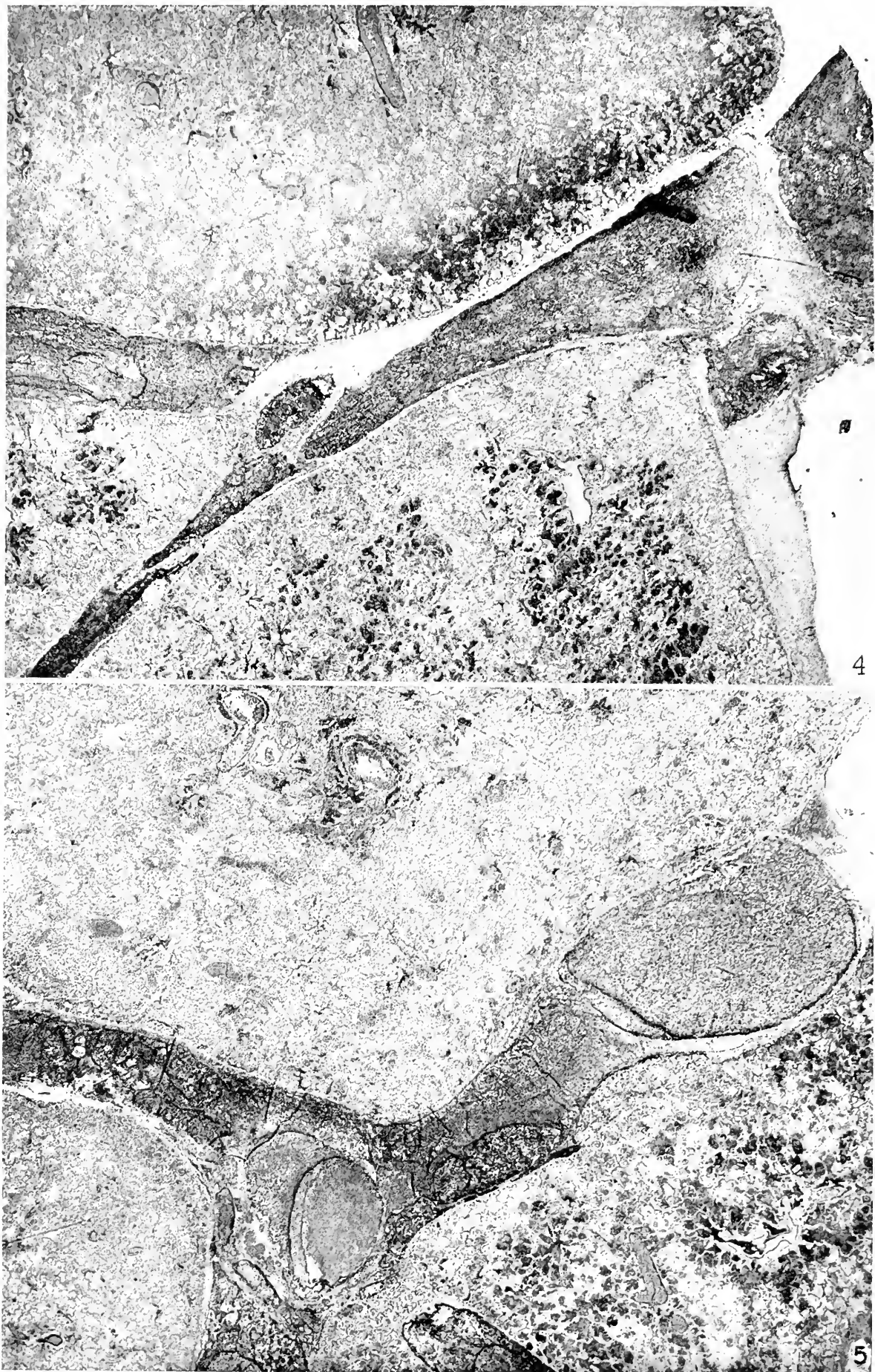
2



3

(Jones and Little: Pneumonia in dairy cows.)

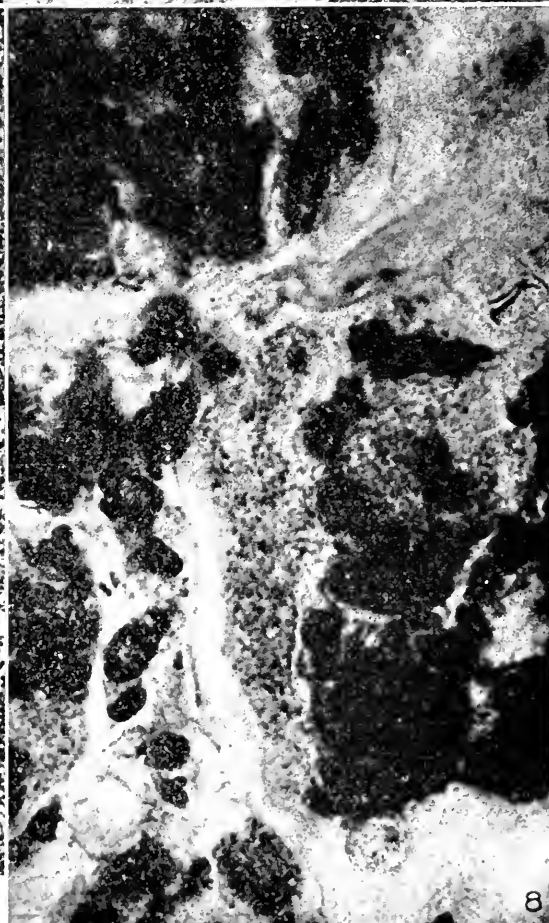
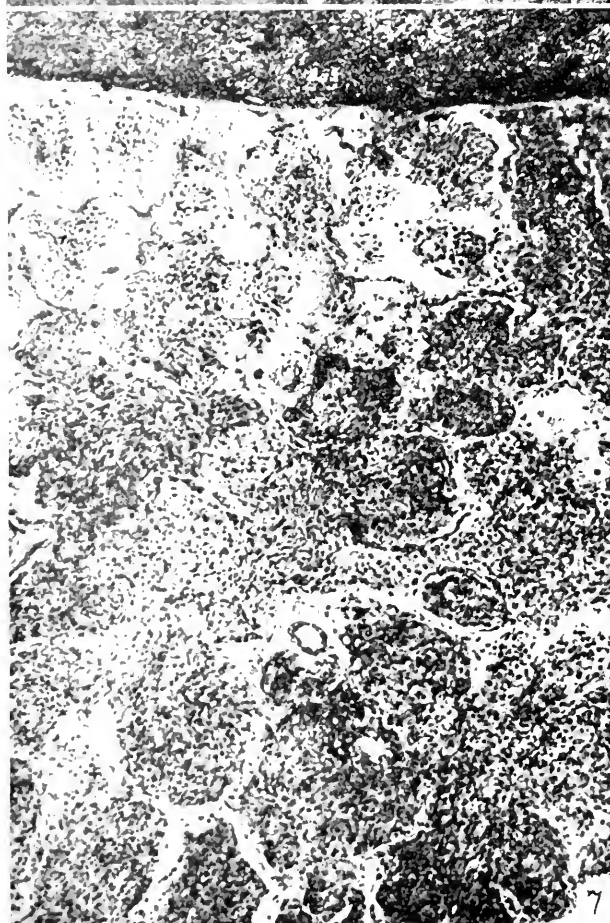
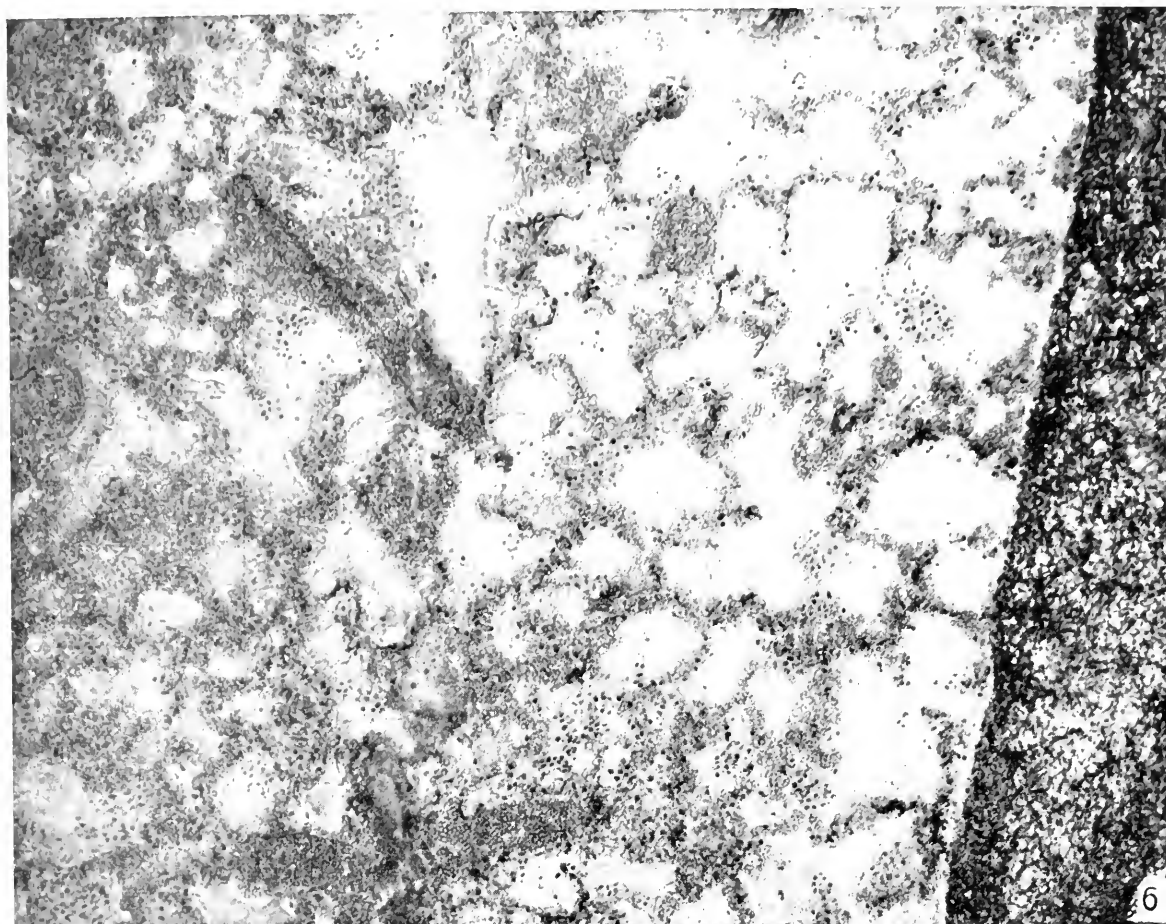




(Jones and Little: Pneumonia in dairy cows.)







(Jones and Little: Pneumonia in dairy cows.)





## A STUDY OF BACILLUS BOVISEPTICUS.

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Although the hemorrhagic septicemia organisms are well recognized and known to attack many species of animals, the relation of one type to another is not clearly understood. The organisms associated with bubonic plague, swinge-plague, and fowl-cholera have been studied more extensively than those of the bovine species. My attention was aroused by certain differences in cultural characters and agglutination affinities between the organisms isolated from an outbreak of pneumonia in cows<sup>1</sup> and those previously obtained by Theobald Smith<sup>2</sup> from cases of pneumonia in calves.

The literature on the cultural and immunological characters of *Bacillus bovisepcticus* is meager. Much of the work was done before the various substances used in fermentation tests came into use. There are, however, a few descriptions of the fermentative characters of this organism. Early immunological studies consisted of attempts to protect laboratory animals with immune sera against small doses of virulent culture.

Theobald Smith<sup>3</sup> described organisms isolated from cases of bovine pneumonia as short, non-motile, encapsulated rods which failed to grow on potato. They did not change the reaction of milk. Acid production in dextrose and saccharose was constant, but fermentation failed to occur in lactose. Phenol was produced in old peptone broth cultures. The formation of indole was variable. His cultures were pathogenic for rabbits in doses of 0.1 to 0.2 cc. of bouillon cultures. He states that small doses proved fatal for rabbits in from 24 hours to 7 or 8 days. Smith recognized the great similarity existing between members of the hemorrhagic septicemia group. Others have also commented on this point.

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<sup>1</sup> Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1921, xxxiv, 541.

<sup>2</sup> Smith, T., *J. Exp. Med.*, 1921, xxxiii, 441.

<sup>3</sup> Smith, T., *U. S. Dept. Agric., Bureau Animal Industry, 12th and 13th Ann. Rep.*, 1895-96, 119.

Ostertag<sup>4</sup> studied two strains of *B. bovissepticus* and reached the conclusion that this organism was identical with the swine-plague and chicken-cholera organism. Mention is not made of specific characters of any of the species employed.

Schirop<sup>5</sup> studied three strains isolated from pneumonia of calves. These he terms *B. vitulisepticus*, although Poels<sup>6</sup> in 1886 applied the name *vitulicidum*. The calf strains were compared with cultures of *B. avisepticus* and *B. suissepticus*. The bacteria were grown in bouillon containing twelve test substances; seven were carbohydrates, the others were alcohols. The action on dextrose, saccharose, maltose, and mannitol seemed to offer the most definite contrast. Lactose was not used. Two of the calf strains, the swine-plague, and chicken-cholera bacilli possessed similar fermentation characters. They attacked dextrose, saccharose, and mannitol, but did not ferment maltose. The other calf organism fermented only dextrose. All organisms formed indole. The calf organisms were of considerable virulence since doses of 0.000001 cc. of bouillon cultures killed rabbits in 24 hours.

Magnusson<sup>7</sup> observed an outbreak of hemorrhagic septicemia among the reindeer in Lapland. He compared the organism isolated with a strain of *B. bovissepticus* and a culture of *B. avisepticus*, and correlated them with Schirop's *B. vitulisepticus*. Their fermentative characters were tested in fifteen carbohydrate media and six containing alcohols. He was unable to show cultural differences in the three organisms studied. All produced acid in lactose, saccharose, and mannitol. Maltose was not fermented. The organism isolated from the reindeer did not produce indole or cause hemolysis. It possessed considerable pathogenicity for mice, rabbits, and guinea pigs, and a sheep. A calf and a cow injected with cultures reacted with high temperatures for 3 or 4 days but recovered.

Magnusson immunized a sheep with living cultures of the reindeer organism. In all, five injections were given during a period of 96 days. Agglutinins could not be detected in the serum. This serum afforded but slight protection for rabbits and mice against small doses of the homologous culture and cultures of *B. avisepticus* and *B. bovissepticus*.

Besemer's<sup>8</sup> studies of the fermentative characters of ten strains of pasteurella led him to conclude that the members of this group were practically uniform in their biochemical actions. From his tables it will be noted that both strains of *B. bovissepticus*, the two cultures of the chicken-cholera bacillus, *B. suissepticus*, and three cultures of Magnusson's reindeer pasteurella attacked dextrose, saccharose, and mannitol, but did not acidulate media containing lactose or maltose. Two of Magnusson's calf pasteurella differed from the others in their ability to ferment lactose. They also failed to attack maltose.

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<sup>4</sup> Ostertag, R., *Z. Infektionskrankh. Haustiere*, 1908, iv, 1.

<sup>5</sup> Schirop, H., *Centr. Bakt., 1te Abt., Orig.*, 1908, xlvii, 307.

<sup>6</sup> Poels, J., *Fortschr. Med.*, 1886, iv, 388.

<sup>7</sup> Magnusson, H., *Z. Infektionskrankh. Haustiere*, 1914, xv, 61.

<sup>8</sup> Besemer, A. M., *J. Bact.*, 1917, ii, 177.

It will be seen from the limited available literature that *B. bovissepticus* and its allied organisms, such as those isolated from calves and reindeer, are regarded as a type which cannot be clearly differentiated from each other or from other members of the hemorrhagic septicemia group. A careful study of the tables of the various observers indicates that such is not the case. The fermentation of lactose by certain of Magnusson's and Besemer's strains is indicative of cultural differences.

References in regard to the application of the agglutination test as an aid in classifying *B. bovissepticus* and the allied pasteurella have not been found. Magnusson failed to detect agglutinins in the blood of a sheep immunized with the reindeer organism. Schirop makes no mention of agglutinins in the blood of rabbits immunized with the calf organisms. Hadley,<sup>9</sup> on the other hand, immunized a rabbit with *B. avissepticus*. The serum of this rabbit agglutinated the homologous strain at a dilution of 1:160. He regarded such a serum as of slight value in testing a doubtful strain.

#### EXPERIMENTAL.

It has been possible to show that certain well defined cultural and serological groups exist among the strains of what may be termed the *bovissepticus* group. The source of the cultures together with a brief note of the conditions from which they were obtained is given in Table I. Mention has been made that these organisms have fallen into distinct cultural and morphological groups. For convenience they have been arranged in Table I according to their specific grouping, which will be brought out in other tables.

Table I indicates that Strains 530, 533, and 537 were isolated from severe cases of pneumonia in cows during the small outbreak previously described. The disease was confined at first to the cows, but later spread to the calves. Cultures 550 and 562 were isolated from calves following the outbreak among the cows. The outbreak subsided but sporadic cases have occurred from time to time in both the dairy herd and young stock. Cultures 578 and 583 are from such sources. Strain 529 was isolated from a calf at the close of another small outbreak on a farm on Long Island.

Strains 6, 209, 432, 436, 558, and 9 were isolated by Theobald Smith from calves during his studies on *Bacillus actinoides*. The calves belonged to the herd in which the outbreak of acute pneumonia

<sup>9</sup> Hadley, P., *J. Bact.*, 1918, iii, 277.

TABLE I.  
*Source of the Strains Studied.*

Strain No.	Date of isolation.	Source.	Type of disease encountered.	Organisms other than <i>B. bovissepticus</i> .
530	Nov. 12, 1920	Outbreak among cows on nearby farm (A).	Diffuse pneumonia; virus localized within lungs and chest cavity.	None.
533	" 14, 1920			
537	" 19, 1920			
550	Dec. 19, 1920	Calf, at close of epidemic on Farm A.	Nodular, necrotic pneumonia.	<i>Staphylococcus albus</i> .
562	Jan. 26, 1921	Calf, apparently a sporadic infection, Farm A.	Diffuse pneumonia.	None.
578	Apr. 1, 1921	Cow, exposed during outbreak on Farm A.	Nodular, necrotic pneumonia.	"
583	Mar. 23, 1921	Calf, Farm A.	Apparently umbilical infection; septicemia with pneumonia.	"
529	Nov. 8, 1920	" farm on Long Island.	Focal consolidation in right caudal lobe; necrotic nodule in left caudal lobe.	Spirillum and spore-bearing bacilli.
6	Jan. 15, 1917	" small outbreak in 1917, Farm A.	Complete consolidation of anterior portion of the right cephalic lobe; nodular areas in the posterior half; purulent exudate in trachea and bronchus.	None.

209	Mar. 5, 1918	Calf, Farm A.	Diffuse pneumonia.	<i>B. actinoides</i> .
432	Oct. 21, 1919	" "	"	" " and <i>B. pyogenes</i> .
436	Nov. 4, 1919	" "	"	Serum-liquefying organism.
502	May 8, 1920	" lung obtained from abattoir in New York City.	Gray to red consolidation of right cephalic lobe.	None. <i>B. necrophorus</i> in liver.
558	Jan. 1, 1921	Calf, Farm A.	Diffuse pneumonia.	<i>B. actinoides</i> .
Bov. Pn. I	About 1913	" Mass.	Necrotic "	None.
9	Feb. 17, 1917	" Farm A.	Diffuse "	<i>B. actinoides</i> and cocci.

occurred in the adult cows. All strains except No. 558 were obtained before cases of *bovisepticus* pneumonia began to appear among the adult cows. In the main, as shown by Theobald Smith, these organisms were associated with pneumonias in calves attributed to a primary infection with *Bacillus actinoides*. In the instance of Calf 558 but few colonies of *Bacillus bovisepticus* were noted. *Bacillus actinoides* was present in great numbers. Culture 502 was isolated from a calf's lung obtained from an abattoir in New York City. The only available history for Culture Bovine Pneumonia I is given in Table I. Strain 9 was associated with *Bacillus actinoides*.

Morphologically all the organisms resembled each other. None were motile; all were Gram-negative.

#### *Fermentation and Other Tests.*

It soon became apparent that a study of the fermentative characters would assist in dividing the strains into cultural groups. Tubes of fermented bouillon containing 1 per cent of dextrose, lactose, saccharose, maltose, mannitol, and salicin were used to test acid production. At first fermentation tubes were employed, but since none of the strains produced gas, ordinary tubes were substituted. Titrations and hydrogen ion concentrations were determined after 7 days incubation at 38°C. The results of these and other tests are given in Table II. Inulin was also used as a test substance. None of the organisms fermented it.

It will be noted from Table II that the organisms fall into three distinct groups. The largest group comprises all strains obtained from the adult cows in the outbreak of pneumonia (Nos. 530, 533, and 537) and other infections (Nos. 550, 562, 578, and 583) occurring in the adults and calves following this outbreak. Culture 529, isolated from a calf at the close of a small outbreak in another state, likewise falls into this group. Members of Group I attack dextrose, saccharose, maltose, and mannitol to about the same degree. The final hydrogen ion concentration varies between pH 6.1 and 6.5. Lactose is also attacked but to a less degree. After several transfers on artificial media all members of this group ferment lactose to the same extent as the other substances. This has been brought out

clearly in Table III. Salicin is not fermented. It will be noted that these strains do not produce indole. Retests after several months on artificial media have confirmed this point. They are not soluble in ox or guinea pig bile. In agar plates containing 8 per cent of defibrinated horse blood the colonies resemble those of the bovine streptococci. The surface colonies are round, flattened, and trans-

TABLE II.  
*Cultural Characters of Sixteen Strains of B. bovissepticus.*

Strain No.	Dextrose.		Lactose.		Saccharose.		Maltose.		Mannitol.		Salicin.		Indole.	Hemolysis.	Bile solubility.
	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH			
	per cent		per cent		per cent		per cent		per cent		per cent				
530	2.6	6.2	1.8	6.7	2.7	6.2	2.9	6.2	2.7	6.3	1.2	7.6	—	+	—
533	3.6	6.2	1.7	6.8	3.4	6.3	2.8	6.2	2.6	6.3	1.0	7.5	—	+	—
537	2.7	6.1	1.8	6.7	2.4	6.4	2.7	6.2	2.6	6.2	0.9	7.5	—	+	—
550	3.0	6.1	1.6	6.8	2.4	6.2	2.6	6.3	2.6	6.3	1.0	7.5	—	+	—
562	2.5	6.3	1.9	6.6	2.6	6.3	2.6	6.3	2.5	6.4	0.9	7.5	—	+	—
578	2.8	6.2	2.6	6.4	2.8	6.4	3.1	6.2	2.7	6.3	1.0	7.5	—	+	—
583	2.9	6.1	2.5	6.5	3.3	6.1	3.0	6.3	2.9	6.2	0.9	7.9	—	+	—
529	2.5	6.2	1.7	6.7	2.7	6.4	2.8	6.4	2.5	6.5	1.3	7.3	—	+	—
6	2.5	6.3	0.2	7.9	2.2	6.4	0.6	7.8	0.5	7.9	0.9	7.5	+	+	+
209	2.5	6.3	1.0	7.4	2.6	6.3	1.3	7.6	1.3	7.5	1.4	7.7	+	—	+
432	2.6	6.3	0.6	7.6	2.5	6.4	0.8	7.5	1.0	7.6	1.1	7.4	+	—	+
436	2.4	6.5	0.9	7.5	2.0	6.6	1.0	7.5	1.0	7.5	1.0	7.5	+	—	+
502	2.7	6.1	1.0	7.3	2.7	6.4	1.1	7.5	1.0	7.5	1.0	7.5	+	—	+
558	2.8	6.3	1.0	7.4	2.7	6.2	1.0	7.5	1.0	7.5	1.0	7.4	+	—	+
Bov. Pn. I	2.8	5.9	0.6	7.4	2.7	6.1	0.7	7.5	2.8	6.1	1.2	7.8	+	—	—
9	2.9	5.8	1.0	7.8	2.5	5.9	1.1	7.8	2.5	6.2	0.9	7.5	+	—	—

lucent. They usually attain a diameter of 3 to 5 mm. after 48 hours. The deep colonies are ovoid, and after 24 hours they are surrounded by a clear zone of hemolysis which tends to widen a little during the next 24 hours of incubation. All Group I strains are encapsulated.

The next group comprises six members, Nos. 6, 209, 432, 436, 502, and 558. They ferment dextrose and saccharose. Lactose, maltose, mannitol, and salicin are not attacked. At first some of these strains produced only small amounts of acid in saccharose.

In two instances it was necessary to add a few drops of sterile serum to the medium to insure maximum acidity. TenBroeck<sup>10</sup> was able to show that serum failed to hydrolyze saccharose, so that it appeared safe to use it to enrich this medium. In the bulb of the fermentation tube broth containing lactose, maltose, and mannitol becomes more alkaline. All members of this group produce indole. They do not hemolyze horse blood in agar plate cultures. The surface colonies rarely reach 2.5 mm. in diameter. They are round, flattened, and more opaque than those of Group I. The deep colonies may be surrounded by a hazy green discoloration. Members of Group II resemble those of Group I in morphology. All are encapsulated. When a few drops of ox or guinea pig bile are added to small amounts of 24 hour bouillon cultures of Group II organisms the fluid commences to clear at once. In most instances after 5 or 10 minutes the mixture becomes limpid. One culture was more resistant but cleared within an hour.

Strains Bov. Pn. I and 9 differ from the preceding group in their ability to ferment mannitol in addition to dextrose and saccharose. In media containing lactose, maltose, and salicin, acid is not produced. Both strains produce alkali in the bulb of the fermentation tube in broth containing lactose, maltose, and salicin. Indole is formed by both. The colonies in horse blood agar plate cultures resemble those of Group II. Hemolysis was not observed. Both strains are insoluble in ox or guinea pig bile. Capsules could be demonstrated in Culture Bov. Pn. I, but not in the case of Culture 9.

*Virulence.*—Many have commented on the pathogenicity of *Bacillus bovissepticus* for rabbits, guinea pigs, and mice. Evidently considerable variation in virulence exists among these organisms. Among the Group I cultures all have been of relatively low virulence for rabbits and mice. 0.25 cc. of a freshly isolated 24 hour bouillon culture injected subcutaneously into rabbits produced only slight local reactions. Mice survived when injected beneath the skin with 0.1 cc. of bouillon culture. After a few generations on artificial media, mice are not killed with doses of 0.5 cc. of bouillon culture administered into the peritoneal cavity. A calf when injected into

<sup>10</sup> TenBroeck, C., *J. Exp. Med.*, 1920, xxxii, 345.



the subcutis of the neck with pleural exudate from a cow dead of the disease developed only a slight local reaction. 10 cc. of bouillon culture introduced through the walls of the trachea of another calf failed to produce ill effects. It was pointed out in a previous communication that once these organisms gained a foothold within the lung they were capable of producing a severe diffuse pneumonia.

None of the organisms of Group II were of particular virulence. Doses of 0.25 cc. of a 24 hour bouillon culture injected subcutaneously into rabbits produce a moderate local reaction which may be absorbed or give rise to a local abscess. Mice are no more susceptible to injection with Group II organisms than with those of Group I.

TABLE III.  
*Fermentation of Lactose by the Organisms of Group I.*

Strain No.	Lactose broth.		Fermented broth.	
	Titration.	pH	Titration.	pH
	<i>per cent</i>		<i>per cent</i>	
530	2.9	6.2	0.9	7.8
533	2.8	6.2	0.9	7.7
537	2.8	6.3	0.9	7.6
550	3.0	6.2	1.0	7.7
562	3.0	6.1	0.9	7.7
529	2.9	6.2	0.9	7.7
578	2.6	6.4	1.2	7.6

Culture Bov. Pn. I, although isolated during 1913, has retained considerable virulence. 0.25 cc. of a 24 hour bouillon culture kills rabbits of 2,000 to 3,000 gm. in 3 to 4 days. The organisms are present in the circulating blood 24 hours before death.

It will be observed in Table II that organisms belonging to Group I attacked lactose to a less degree than they did the other substances. In some instances it was doubtful whether fermentation had really occurred. This was particularly true when the organisms were freshly isolated. To decide this point definitely, fermented bouillon of a different lot than that employed in the first observation was tubed in 10 cc. amounts and sterilized as usual. To one-half of the tubes 1 cc. of a sterile 10 per cent aqueous solution of lactose was

added. A tube of fermented bouillon and a tube of the same medium to which the lactose had been added were inoculated with each strain. After 7 days incubation, the tubes were titrated and the hydrogen ion concentration was determined. The results are given in Table III.

There seems to be no doubt that the organisms attack lactose, since they do not produce acid in the fermented broth. Litmus milk cultures change from blue to purple after 48 hours. Further color changes were not noted after 2 weeks of incubation.

A number of strains from Groups I and II were tested for their resistance to drying. Growth from the lower portions of the slants of 24 hour agar cultures were smeared on sterile pieces of cover-slips. The preparations, which dried rapidly in the air, were stored in sterile Petri dishes at room temperature. At regular intervals the cover-slips were dropped into sterile bouillon. One organism survived 18 hours under these conditions. None were alive after 24 hours.

#### *Immunological Tests.*

Since it was possible to show that the organisms usually referred to as *Bacillus bovisepcticus* really belonged to three well recognized cultural groups, it was decided to test their serological relationship. With this in view, rabbits were immunized with a single strain chosen from each group. Much of the previous immunological work with members of the pasteurella group has consisted in attempts to produce a protective antiserum. The results of immunological relationships were judged on the amount of protection afforded against various doses of living culture. Mention is not made of the formation of agglutinins for *Bacillus bovisepcticus* except by Magnusson, who was unable to demonstrate them in the blood of a sheep immunized with the reindeer organism.

That agglutination affords a ready method of grouping organisms is well recognized. The following protocol of Rabbit 1 immunized with Group II, Strain 432, indicates the difficulty with which agglutinins are produced. This is particularly true for Groups I and II.

*Rabbit 1.*—Weight 3,530 gm.

Jan. 13, 1921. 2.0 cc. of bouillon culture of No. 432 heated at 60°C. for 30 min., intraperitoneally.

" 14. 4.0 cc. of bouillon culture of No. 432 heated at 60°C. for 30 min., intraperitoneally.

" 15. 6.0 cc. of bouillon culture of No. 432 heated at 60°C. for 30 min., intraperitoneally.

" 19. 5.0 cc. of killed culture intraperitoneally.

" 20. 6.0 " " " "

" 21. 8.0 " " " " "

" 24. Weight 3,465 gm.

" 26. 10.0 cc. of killed culture intraperitoneally.

" 27. 10.0 " " " " "

" 28. 7.0 " " " " "

Feb. 4. 0.25 " " living 48 hr. bouillon culture subcutaneously.

" 5-6. Local reaction with fever.

" 8. 0.5 cc. of living culture subcutaneously.

" 9. No reaction.

" 12. 1.0 cc. of living culture subcutaneously. Local reaction. Weight 3,463 gm.

" 15. 0.5 " of 24 hr. bouillon culture intravenously. Severe temperature reaction.

" 16. 0.5 " of 24 " " " " Moderate temperature reaction.

" 17. 0.6 cc. of 24 hr. bouillon culture intravenously. Severe temperature reaction.

" 24. Small test bleeding, partial agglutination at 1:100 dilution.

1.0 cc. of 24 hr. bouillon culture intravenously. Temperature reaction.

" 25. 1.0 " " 24 " " " " " "

" 26. 1.0 " " 24 " " " " " "

Mar. 1. Animal sick; dietary disturbance; food withheld for 24 hrs.

" 3. 2.5 cc. of killed culture intraperitoneally.

" 4. 1.0 " " living " intravenously. Temperature reaction.

" 8. Weight 3,328 gm.

" 9. 0.8 cc. of living culture intraperitoneally.

" 10. 2.0 " " " " "

" 12. 2.0 " " " " "

" 15. Test bleeding, agglutination at a dilution of 1:200.

2.0 cc. of living culture intraperitoneally.

" 16. 4.0 " " " " " Weight 3,182 gm.

" 24. Bled. Serum gives agglutination at a dilution of 1:200.

Culture Bov. Pn. I of the third group possesses considerable virulence for rabbits but it is a relatively simple matter to produce a good agglutinating serum with the killed organism. A serum which caused clumping of the homologous strain at a dilution of 1:500 was obtained after the injection of killed cultures over a period of 31 days. It has been possible by further treatment with killed cultures to increase the titer of this serum to 1:5,000.

Antigens prepared by suspending the growth from 24 or 48 hour slant agar cultures in 0.85 per cent sodium chloride solution which contained 0.25 per cent phenol gave the best results. Suspensions having a density of 2.5 with the Gates<sup>11</sup> apparatus were employed. All tubes were incubated at 38°C. for 18 hours. Agglutination of the organisms of Groups I and II takes place slowly. No clumping is visible before the 6th or 8th hour. Both strains of Group III agglutinate much more rapidly, gross clumping begins almost at once and most of the organisms fall to the bottom within 5 or 6 hours.

The results of the agglutination tests of all strains with the sera of three rabbits, each immunized with a separate group strain, are given in Table IV.

Table IV indicates that the immunological grouping with one exception is identical with the cultural grouping. All of Group I are agglutinated by the Group I serum from a rabbit immunized with a single strain. This serum does not cause clumping of the organisms belonging to the other groups. The same holds true when a Group II organism was used in immunization. The Bov. Pn. I antiserum acts only on the organism possessing identical cultural characters. It will be noted that each strain, with the exception of Culture 502, agglutinates well up to the limits of the serum.

In addition to the organisms appearing in the table, one strain of *Bacillus avisepticus* and two cultures of *Bacillus suisepcticus* were tested. The chicken-cholera organism possessed similar cultural characters as those of Group II, but failed to agglutinate at the lowest dilution (1:10) with Group II serum. This organism gave agglutination in the lower dilutions with Group III serum. The swine-plague bacilli were culturally indistinguishable from Group III. One gave a partial agglutination at a dilution of 1:100 with Group III serum. The other failed to agglutinate with either Group II or III serum.

Strain 502 evidently falls into a separate immunological group. That organisms having similar morphological and cultural characters may differ in their immunological properties has been shown clearly by Dochez and Gillespie<sup>12</sup> for the pneumococcus.

<sup>11</sup> Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.

<sup>12</sup> Dochez, A. R., and Gillespie, L. J., *J. Am. Med. Assn.*, 1913, lxi, 727.

TABLE IV.  
*Results of the Agglutination Tests of All Strains against Cultural Group Sera.*

Strain No.	Rabbit immunized with Group I Culture 530.						Rabbit immunized with Group II Culture 432.						Rabbit immunized with Group III Culture Bov. Ph. I.						
	Serum dilutions.						Serum dilutions.						Serum dilutions.						
	1:10	1:20	1:50	1:100	1:200	1:500	1:10	1:20	1:50	1:100	1:200	1:500	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000
530	C.	C.	C.	C.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
533	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
537	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
550	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
562	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
578	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
583	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
529	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
209	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
432	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
436	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
502	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
558	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bov. Ph. I	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	"	"	"	"	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

C. indicates complete agglutination; +, a heavy clumping without entire clearing of the fluid; ++, a moderate agglutination; +, a small but distinct deposit at the bottom of the tube; —, negative reaction.

Results somewhat similar are recorded by Schirop. He immunized rabbits with three different cultures of *Bacillus vitulisepticus*. Two strains were culturally identical. The serum from each rabbit protected mice against the homologous culture but failed to protect against the others.

Each type of serum was absorbed with a single homologous group strain. The sera were then tested against all of their respective group cultures. It was found that the agglutinins had been absorbed uniformly for all.

#### DISCUSSION.

Although the cultures studied were largely obtained from the cows and calves in one herd, yet it has been possible to differentiate them clearly into three cultural and immunological groups. A study of the various data submitted in the tables reveals some interesting points. The herd from which most of the cultures were obtained has been under observation by various members of the staff since 1917. On February 17, 1917, the single Group III strain was isolated from a calf. Since that time similar organisms have not been met with. During January, 1917, a Group II strain was isolated by Dr. Smith. In 1918, 1919, and 1920, he found that organisms of this class were associated with pneumonia in calves, but they have not been regarded as primary agents. This type has become endemic, pure infections with this organism have not been observed. During October, 1920, a third type of *Bacillus bovisepiticus* (Group I) was introduced into the herd. An outbreak of pneumonia among the cows followed in which this organism was the only one obtained. Since the outbreak subsided sporadic cases have been found in the calves and occasionally in cows. Whether Type I will finally displace Type II and in turn be displaced by a freshly introduced virus can only be proved by future observation.

Several other points are worthy of emphasis. The marked differences in cultural characters between the Group I organisms and those of the other groups seem to preclude the name *Bacillus bovisepiticus* for two such markedly different types. That Groups II and III differ so little culturally that they may be considered varieties of the same species seems reasonable. Group I, however, differs to such

a degree in not only its fermentative characters but in its ability to hemolyze and its failure to produce indole that it is hardly possible to consider it a variety of the others.

The Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types<sup>13</sup> includes members of the hemorrhagic septicemia group under the tribe Pasteurellæ, genus *Pasteurella*. The genus is characterized as aerobic or facultative, the powers of carbohydrate fermentation are slight, and no gas is produced. Gelatin is not liquefied. The organisms are parasitic. Nothing exists in this characterization to exclude any of the organisms described from the genus *Pasteurella*. Perhaps the name *Pasteurella* "*bovissepticus*" might be applied to members of Group I. More definite characterization for these organisms would consist in, aside from morphology and capsules which are common to all groups, the ability to produce acid in dextrose, lactose, saccharose, maltose, and mannitol; their inability to produce indole; their insolubility in ox or guinea pig bile; and their power to hemolyze red cells in agar plate cultures. The specific name *vitulisepticus* (Kitt<sup>14</sup> and Schirop) might be applied to organisms of the second group, which may be characterized as follows: acid production in dextrose and saccharose but not in lactose, maltose, mannitol, and salicin, and the production of alkali under suitable conditions in certain media. Indole formation is constant, but none produce hemolysis in horse blood agar plate cultures. All members of this group are soluble in ox and guinea pig bile. For the present, Group III may be regarded as a variety of Group II, since they differ culturally in but two details: their ability to attack mannitol as well as dextrose and saccharose and their insolubility in ox and guinea pig bile.

Since these data have been gathered together another organism has been isolated from a cow which developed septicemia from a wire which had penetrated from the rumen through the diaphragm and into the heart. This organism attacks the same substances as the Group I strains, but it is not hemolytic and it produces indole. It is reasonable to suppose that if a large number of cultures from various sources were studied more cultural groups would be found.

<sup>13</sup> Winslow, C.-E. A., Broadhurst, J., Buchanan, R. E., Krumwiede, C., Rogers, L. A., and Smith, G. H., *J. Bact.*, 1920, v, 191.

<sup>14</sup> Kitt, cited by Schirop.<sup>5</sup>

The immunological findings are of considerable interest. It has been shown that at least three serological groups exist. From present indications it would appear that organisms having the same cultural characters do not necessarily have similar agglutination affinities. This is true of Strain 502, which is a typical Group II organism but which fails to agglutinate with its group serum. Doubtless the study by others of strains isolated from different sources may throw more light on this point. This has been noted when a few species from other hosts have been tested. The chicken-cholera organism which agreed with Group II in cultural characters failed to agglutinate with this serum but did agglutinate with Type III serum. Two swine-plague cultures were identical culturally with the third type, one agglutinated moderately with Group III serum, the other was not agglutinated. It may be possible to show immunological differences between the pasteurella attacking different species. Perhaps the agglutination test will afford a rapid and efficient method for the differentiation of the human plague bacillus from members of the hemorrhagic septicemia group which attack the lower animals. These immunological differences must be taken into account when attempts are made to control infections in the field.

#### SUMMARY.

It has been possible to study the cultural characters and certain of the immunological relationships of sixteen strains of *Bacillus bovisep-ticus*. The organisms have fallen into three distinct cultural groups. The largest group comprises eight strains, four of them obtained in pure cultures from cases of pneumonia in cows, two others were pure infections in calves, the others were associated with other organisms. This group may be characterized as short, non-motile, Gram-negative, encapsulated rods which fail to produce indole but produce clear zones about the deep colonies in horse blood agar plate cultures. Members of this group produce acid in media containing dextrose, lactose, saccharose, maltose, and mannitol. The final hydrogen ion concentration in dextrose broth lies between pH 6.3 and 6.1.

The next largest aggregation comprises six strains. These organisms ferment dextrose and saccharose, but do not attack lactose,



maltose, or mannitol. All produce indole. All are soluble in ox and guinea pig bile but none are hemolytic.

The other two strains resemble the preceding but attack mannitol in addition to dextrose and saccharose. They produce indole but are not bile-soluble.

In the main the immunological relationship has been found to follow cultural grouping. All of Group I are agglutinated by a serum prepared by immunizing a rabbit with a single Group I strain. This serum fails to agglutinate members of other groups. The same holds true with Group II and III sera. In no instance has cross-agglutination between groups taken place.



# THE FACTOR DETERMINING THE SPREAD OF RED MARROW DURING ANEMIA.

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PLATES 47 TO 49.

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It has long been known that in certain forms of anemia the red marrow extends throughout the bones, whereas in other forms equally chronic, little if any marrow spread occurs. The cause for this striking difference is not clear. Some experiments here to be detailed bear on the problem thus presented.

Several observers have noted that blood regeneration takes place less rapidly after hemorrhage than when a destruction of corpuscles has occurred within the body.<sup>1,2</sup> The extraordinarily rapid improvement of cases of pernicious anemia after the so called blood crises, as well as the active regeneration of erythrocytes which follows hemolysis by poison, have long excited comment. Itami and Pratt<sup>2</sup> found that rabbits rendered anemic by destruction of red cells within the body recover much faster than control animals deprived of blood by hemorrhage; and Robertson<sup>3</sup> showed that animals rendered plethoric by transfusion recovered with extraordinary rapidity from anemia due to intravascular blood destruction. An obvious explanation of these diverse facts is to be found in the conservation within the body of the products of the destroyed cells, and it has been shown that injections of laked blood will hasten regeneration after bleeding.<sup>4</sup> What is the part played by the stroma substances in this recovery? May one perhaps deplete the organism of them and thus lead to the production of fragile cells, similar possibly to the cells that are characteristic of hemolytic icterus? Our experiments were devised originally to answer this question. Animals were bled repeatedly and

<sup>1</sup> Ritz, H., *Folia hæmatol.*, 1909, viii, 186.

<sup>2</sup> Itami, S., and Pratt, J., *Biochem. Z.*, 1909, xviii, 302.

<sup>3</sup> Robertson, O. H., *J. Exp. Med.*, 1917, xxvi, 221.

<sup>4</sup> Itami, S., *Arch. exp. Path. u. Pharmacol.*, 1910, lxii, 104.

the hemoglobin was replaced in the hope that blood regeneration would thereby be so greatly enhanced as to exhaust the store of stroma substances.

Four series of twelve rabbits each were rendered anemic by almost daily bleedings from the heart during a period of 6 to 8 weeks. Prior to the first bleeding the animals were kept under observation for several days and repeated hemoglobin estimations were made by the Palmer method, and the resistance of the cells to hypotonic salt solution was determined. On the basis of the findings, the animals were grouped into pairs having similar weights and hemoglobin readings; and repeated bleedings from the heart were begun by an aseptic technique. One individual of each pair received a solution of concentrated hemoglobin immediately after each bleeding by subcutaneous injection into the anterior and lateral abdominal walls, care being taken to avoid the veins. The injected pigment was distributed in the tissue by gentle massage. The hemoglobin solution was prepared by the method of Sellards and Minot<sup>5</sup> from fresh rabbit blood obtained by cardiac aspiration, and was made up with salt solution to a percentage (140 per cent, Palmer) somewhat in excess of that found in the blood. The amount injected, 5 to 7 cc. each time, or slightly more pigment than was removed at a single bleeding, was rapidly absorbed. Usually a slight reddish stain alone remained at the end of 24 hours and there was never any noticeable tissue reaction.

To avoid introduction of the factor of malnutrition which is known to depress marrow activity (Naegeli), the bleedings were so proportioned as to cause only a mild anemia. The weight was carefully followed and usually a gain was noted during the experiment. 10 cc. of blood was taken (in large animals 15 cc.) every day until the hemoglobin reading had fallen to just below 50 per cent. At this approximate level it was maintained. Any rabbit showing a less amount of hemoglobin was not bled until regeneration had again brought the pigment above 50 per cent. As the rabbits had, to begin with, hemoglobin readings of only 75 to 100 per cent, it will be seen that the degree of anemia induced was not great. The individuals given injections of hemoglobin received it every week-day, whether bled or not. After 2 or 3 weeks of bleedings and injections, they regularly formed hemoglobin much more rapidly than the controls, as shown by the fact that far more blood had to be taken from them to keep their hemoglobin at the 50 per cent mark.

The resistance of the red cells to hypotonic salt solution was taken as the index to their fragility. It was determined frequently according to the usual technique. From time to time a color index was obtained, that is to say the ratio of hemoglobin to total cell bulk in a given amount of blood as determined by Epstein's microhematocrit method.<sup>6</sup> The observations on the blood were always made in the morning and the bleedings and injections followed in order.

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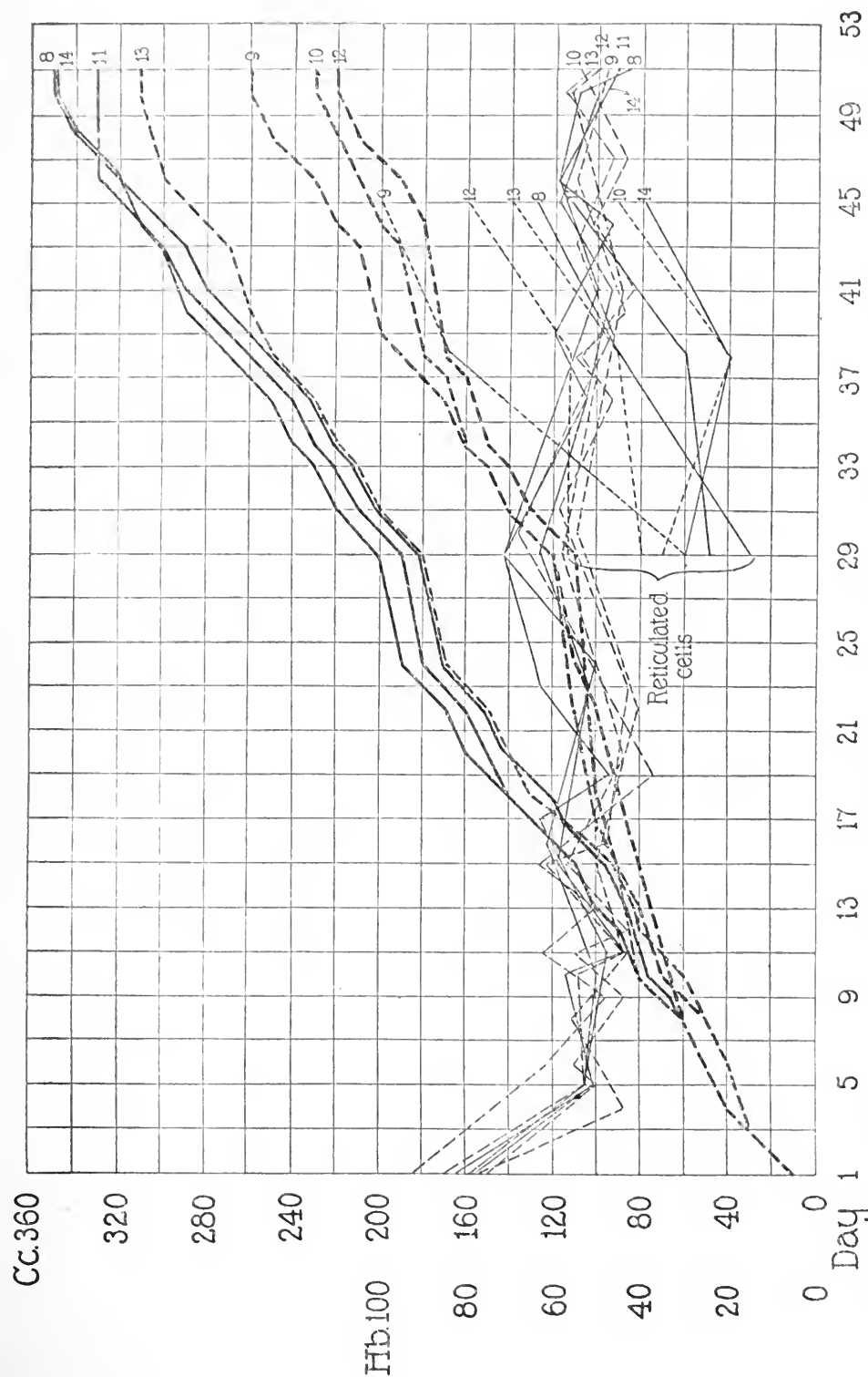
<sup>5</sup> Sellards, A. W., and Minot, G. R., *J. Med. Research*, 1917, xxxvii, 161.

<sup>6</sup> Epstein, A. A., *J. Lab. and Clin. Med.*, 1915-16, i, 610.

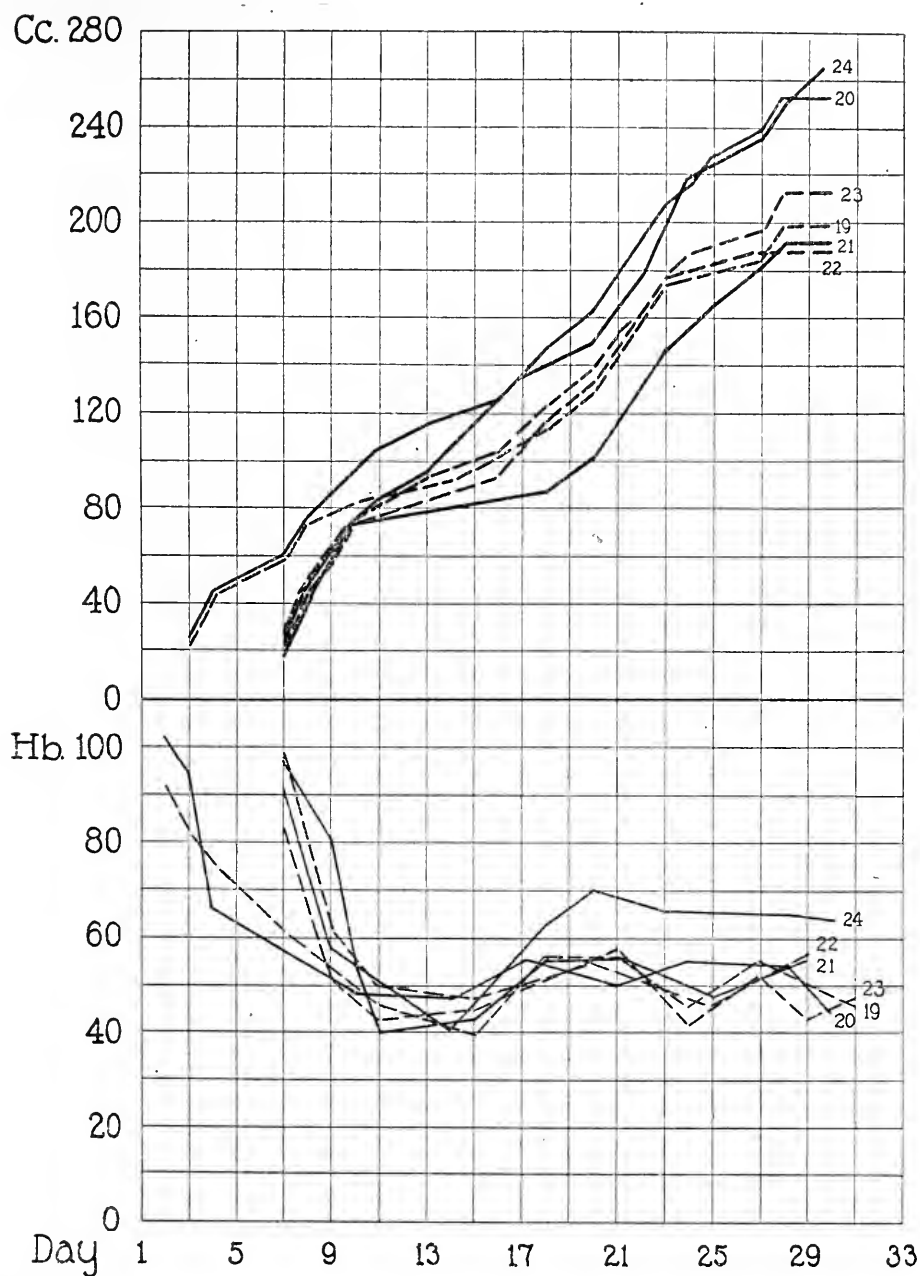
*Blood Changes.*

The most marked difference, manifest during life, between the experimental animals and the controls was the greater rapidity of blood regeneration in the former. This was expected, and save on the assumption of its occurrence our experiments would not have been undertaken, for only through its means could the opportunity for stroma depletion be rendered relatively great in the experimental animals. That the removal of corpuscular substance was far more considerable in them than in the controls is shown by relative amounts of blood removed (Text-figs. 1 to 4), taken in connection with the color index. For this latter, as already stated, the ratio was used, not of hemoglobin to cell number, since the size of the cells may vary, but of hemoglobin to corpuscle volume, that is to say, to actual bulk of hemoglobin-containing tissue. This ratio remained practically the same in the injected and control animals, whence it follows that the depletion recorded in the charts refers not merely to hemoglobin but to total corpuscle substance as well. Though the depletion was far greater in the injected animals, the resistance of their cells gave no indication of a dearth of stroma substance, showing only the same insignificant variations observable in the controls.

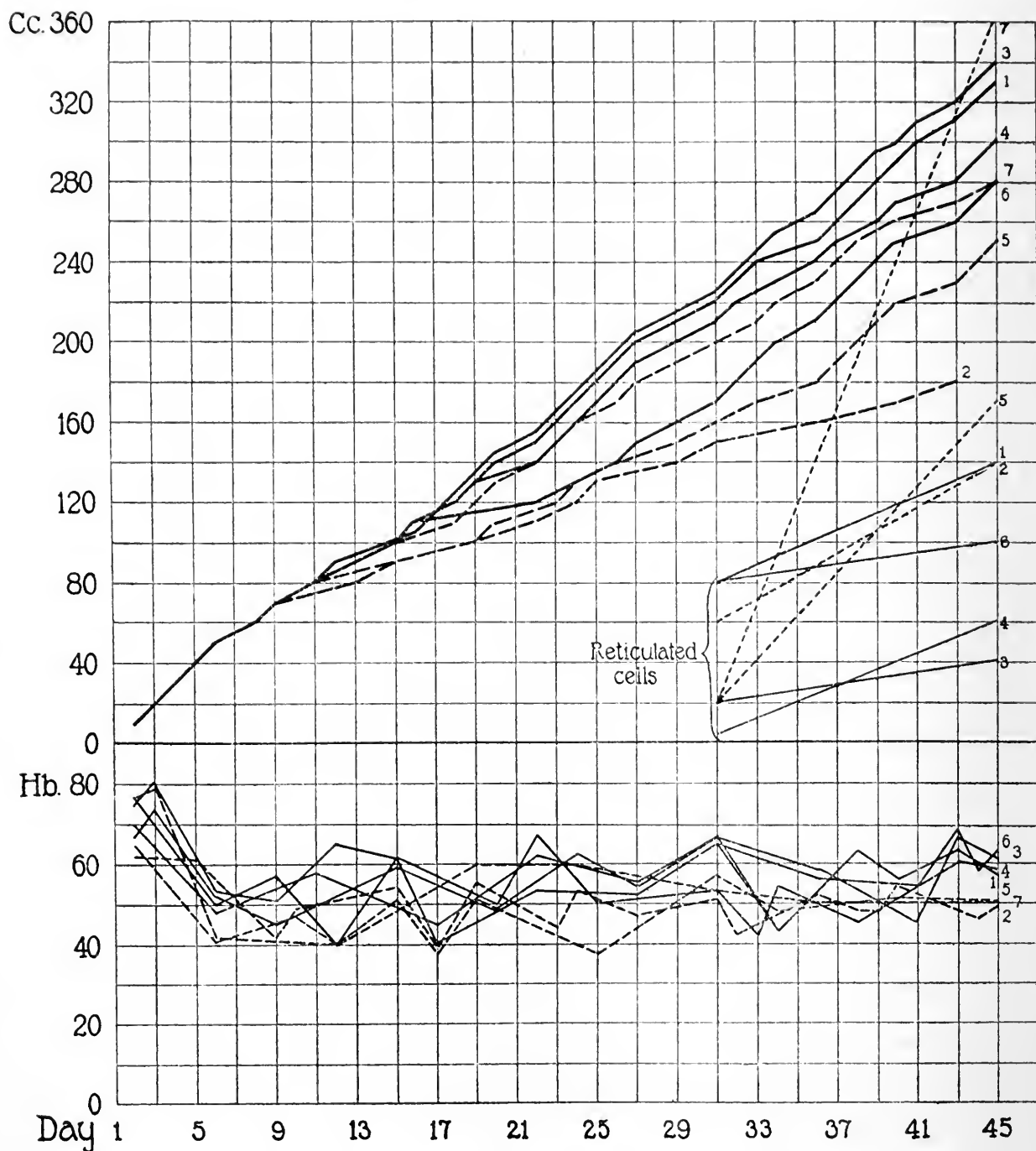
By another clinical method evidence of a stroma lack was sought for. Under the conditions of active regeneration from anemia an increased number of reticulated red cells is found in the blood. Many, indeed most, of these cells as there seen are polychromatophilic and obviously imperfect. It was thought that marrow strain might be relatively great in the injected animals, since they were manufacturing more blood than the controls and had less stroma material to do it with, and that, therefore, a larger proportion of reticulated red cells would be found circulating in them. The exact opposite proved to be the case. Though with the development of anemia the reticulated cells increased in both sets of animals, they were, after the first few weeks, most numerous in the controls and at length strikingly so, as is shown by Text-figs. 1 and 3. Only very occasional nucleated red cells were found.



TEXT-FIG. 1. Illustrating the effect on hemoglobin regeneration of subcutaneous injections of the pigment. There were seven rabbits, three of which (Nos. 8, 11, and 14) received injections while the others served as controls. All were bled to the extent necessary to keep their hemoglobin at approximately 50 per cent (Palmer). In the chart, one set of faint lines shows the individual hemoglobin percentages from the time the bleedings were begun. The heavy lines indicate the mounting totals of blood removed from each rabbit. A second set of faint lines shows the late variations in the number of reticulated red cells in circulation. In order to simplify the chart many of the daily determinations which merely fall in with its general trend have been omitted. It will be seen that much more blood was taken from the injected animals to maintain their anemia at the level set than was necessary in the controls. The difference was not at first noticeable but became marked as time passed. Late in the experiment reticulated red cells were relatively less numerous in the injected animals despite the fact that these were forming more blood than the controls.

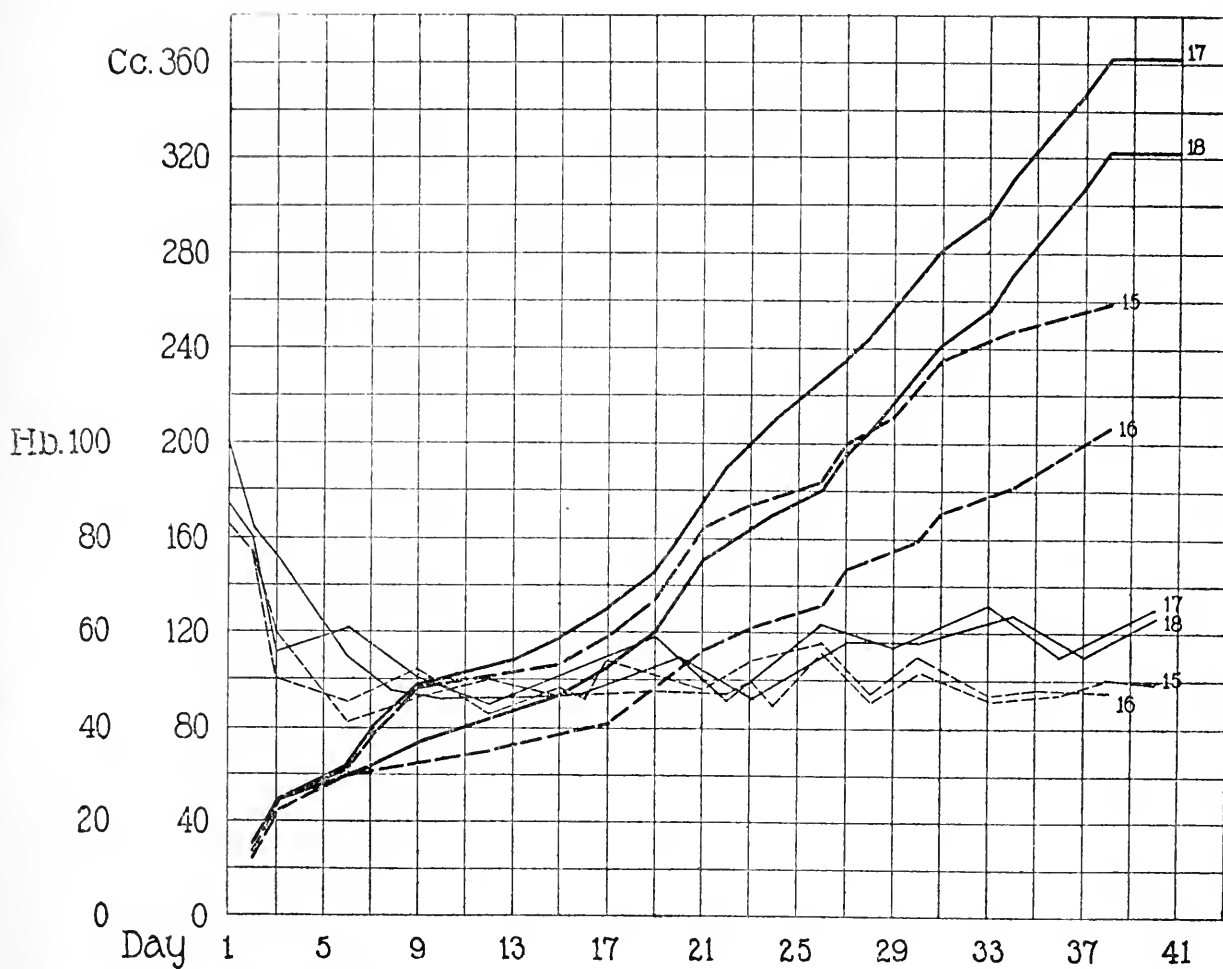


TEXT-FIG. 2. The same general explanation applies here as in Text-fig. 1, and nearly identical findings are illustrated. Rabbits 20, 21, and 24 received the pigment injections. Of these only No. 21 failed to regenerate hemoglobin better than the three controls. The percentage of reticulated cells is not charted.



TEXT-FIG. 3. This figure shows the same facts as those preceding. Despite the large quantities of blood removed from the injected animals (Nos. 1, 3, 4, and 6) they were not rendered quite as anemic as the controls.





TEXT-FIG. 4. In the injected animals (Nos. 17 and 18) of this series, the bleedings were eventually pushed to the point of danger, owing to the repeated sudden reductions in blood volume, yet the percentage of circulating hemoglobin was not prevented from rising above that of the controls.

*Morphological Changes.*

The reason for the differences above noted was strikingly evident at autopsy. The erythropoietic tissue of the animals injected with hemoglobin had undergone extensive hyperplasia, whereas that of the controls had increased little if at all.

The animals were killed with chloroform. Pieces of the liver, kidneys, and spleen were saved to determine whether there had been extramedullary blood formation. It was never notable. All of the large, long bones—femur, tibia, humerus, and ulna—were stripped of muscle and split open along one side to expose the entire length of the marrow cavity. It was early recognized that only by averages might a conclusion be arrived at as regards marrow spread, since the red marrow of normal rabbits varies greatly in extent. Thus, for example, in the femur it may occupy only the upper third of the shaft or practically the whole of it, though in the latter case there is ordinarily a considerable admixture of fatty tissue toward the distal end. In the tibia, a little red marrow is normally present at the proximal end and somewhat less is found in the humerus and ulna. All of the marrows removed from the anemic animals were fixed in Zenker's fluid and sections made,—in some series of the entire marrow length and in others of cross-sections of the proximal, middle, and distal portions.

It cannot be said that in the control animals there was any definite increase of hematopoietic tissue over the normal. Always it was rather pale, and it extended, mixed with more or less fat, throughout the upper one- to two-thirds of the femur, but was never present in any great quantity in the humerus, tibia, and ulna. A far different condition was encountered in the injected animals. Here the entire shafts of femur and humerus were filled with deep red, typical "currant jelly" marrow, and the tibia and ulna contained a similar tissue throughout their length which in the controls held almost nothing but fat. Figs. 1 to 4 illustrate the findings. We would again emphasize the fact that our statement is based not on individual instances, for these vary considerably, but on the assembled findings in 48 rabbits.

The histological pictures as a whole showed a contrast as great as the gross. In the injected rabbits the extension of the red marrow at the expense of the yellow was, of course, well seen, but far more important was a qualitative change in the first mentioned tissue, namely an enormous increase in the erythrocyte-forming elements as

distinct from the leucogenic. Under the microscope large aggregations of the pycnotic nuclei of immature red cells, the mark of erythrogenic islands of great size, at once attracted attention. All the tissue was extremely vascular. A characteristic finding is shown in Fig. 3 and should be contrasted with that of Fig. 4 obtained from the same level in the femur of the control rabbit.

#### DISCUSSION.

The experiments yielded no evidence that rabbits can be depleted of stroma sufficiently to affect the resistance of the red cells to hypotonic salt solution. Rather do they indicate that the elements which go to form stroma can be provided by the body on occasion in quantities enormously in excess of those demanded by any ordinary emergency. This view is supported not only by the blood findings but by the great spread of the erythropoietic tissue in the animals receiving hemoglobin.

All of the rabbits remained healthy and well nourished, and the anemia in all was but moderate. These facts seem to us to rule out the possibility that the injected pigment served merely as a food in the ordinary sense. The diet was mixed and contained much green vegetable, so that an abundance of iron must have been available to the controls. It is possible, of course, that iron compounds readily utilized by the marrow may have been lacking in them and present in the experimental animals by virtue of the injected hemoglobin. But certainly the injections did not bring about any noteworthy short cut in the elaboration of hemoglobin-containing cells. Their effect on the blood did not become clearly evident until during the 3rd week of anemia, as the charts show. Long before this time, after but a few bleedings, corpuscles poor in hemoglobin were circulating in large numbers just as in the controls. Had any considerable amount of the material provided by the injections made its way to the corpuscular tissue by a metabolic route more direct than the normal, its effect should have been evident early in a rapid replacement of the circulating hemoglobin and perhaps in a relatively high color index. A high color index has indeed been recorded during recovery from anemia abruptly produced by a hemolytic poison or

by sudden intercurrent blood destruction in animals long plethoric from transfusion.<sup>3</sup> But in these instances, the index was determined on the basis of cell number, whereas in the present work the total bulk of the cells in a given amount of whole blood has been used instead. The distinction is worthy of stress since the average size of the corpuscles varies much under different conditions.

Late in the experiments when the injected animals were repairing their blood loss far more rapidly than the controls, reticulated cells were less numerous in their circulation—a fact which at first seems an exception to the generally accepted rule that in anemic states the number of reticulated cells bears a rough relationship to the rapidity with which regeneration is going on. But the exception is only an apparent one. The demands for new blood were nearly identical in all of the rabbits, but they were exerted upon very different amounts of marrow; and in those possessing most of this tissue, that is to say the injected animals, repair would inevitably take place most quickly, granting that the blood-forming elements worked at the same rate in all. Had this been the only factor concerned, the number of circulating reticulated cells should have been approximately the same in both sets of animals. But in the injected rabbits there was an additional advantage. Blood repair in them went on so fast that the 24 hours between bleedings was sufficient for recovery to a slightly higher hemoglobin percentage than in the controls. Thus the anemia from day to day was not really quite so severe as in the latter—a fact reflected in some of the charts,—and the demands on the bone marrow were less drastic and elicited the setting-free of fewer reticulated cells.

The fact is well attested that anemia induced by letting blood from the healthy body does not suffice to cause any marked marrow hyperplasia. Some other factor is necessary. This factor, as shown by our experiments, is the presence in the body of hemoglobin in excess of the amount that the existing hematopoietic tissue can utilize. Under the conditions of a moderate anemia of fixed grade, such as we have dealt with, the marrow is supplied from the body with enough hemoglobin to manufacture cells of a certain kind at a certain rate, cells which may be considered as the inevitable product of the interacting conditions. When more hemoglobin is supplied, as in our

injected animals, and the degree of anemia is not altered, the physiologic choice arises of more heavily pigmented cells to be formed by the same tissue or merely a greater quantity of cells of the same sort. The second alternative is the one chiefly taken; and as the output of the marrow increases this tissue itself spreads.

When the clinical and marrow findings in human anemias of different origin are compared, it is seen at once that there are two large classes which correspond in essentials with our rabbits. Included in the one class are pernicious anemia, malarial anemias, anemia from purpura, and that of various hemolytic origins, in all of which the blood is destroyed within the body and more or less conservation and utilization of its constituents may occur with result that the "turn over" of corpuscular material by the combined processes of destruction and restitution becomes abnormally great. In these cases as in our injected rabbits the marrow spreads. And then there are the anemias from hemorrhage, or from iron deprivation, in which as in our control rabbits there exists a lack and the marrow fails to spread, or spreads slightly and slowly if conditions render the elaboration of hemoglobin relatively easy. Anemias due to marrow depression lie, of course, outside both categories.

Repeated reference has been made thus far to a repair by blood quantity *versus* one by blood quality (altered color index). While the former means of repair preponderated in the conditions with which we have had to deal there are undoubtedly states in which the second is of much importance. In pernicious anemia, as Capps<sup>7</sup> has shown, the color index based on hemoglobin *versus* the volume of the massed corpuscles is often higher than normal, showing that the excess of pigment available for blood formation has had the effect of altering the blood tissue in a qualitative direction. But when this is the case the quantitative output tends to be even more markedly affected, as shown by the enormous marrow spread. That the marrow in cases of pernicious anemia can, on occasion, function as its spread would seem to warrant, is proven by the noteworthy rapidity with which blood repair occurs during remissions in the disease.

<sup>7</sup> Capps, J. A., *J. Med. Research*, 1903-04, x, 367.

## SUMMARY.

Rabbits in which a chronic anemia of moderate grade is induced by repeated bleedings repair the hemoglobin loss much more rapidly when given subcutaneous injections of hemoglobin than when this is not the case. But the effect of the injections is not manifest for several weeks, during which many pale corpuscles are put out by the marrow; whence it follows that the introduced pigment does not find its way in quantity direct to the new-formed cells but must follow a more or less roundabout metabolic route, perhaps the same one as that of ordinary iron compounds destined for the blood.

The rapid replacement of the circulating hemoglobin in the injected animals occurs chiefly through an increased production of corpuscular substance having the same color index as that found in uninjected, anemic controls. By color index in this connection is meant the relation of hemoglobin to the volume of the massed corpuscles.

Late in the period of bleedings and hemoglobin injections the demand for stroma for the new-formed blood is far greater than in control animals that have been merely bled, yet the circulating corpuscles show no lessening in resistance to salt solution, such as might perhaps be expected were there a stroma lack. The hematopoietic tissue of the injected animals undergoes an extensive increase—a fact which speaks strongly for the view that the elements out of which stroma is formed are still abundant.

The factor which determines the spread of red marrow during anemia is shown by our experiments to be the presence in the body of hemoglobin, or perhaps of its precursors, in excess of the amount which can be utilized by the marrow already existing. Numerous illustrations in support of the point can be adduced from human pathology. Two will suffice. The widespread "currant jelly" marrow of pernicious anemia is found in an organism rendered anemic but supplied with hemoglobin in excess; while the pale, restricted marrow of cases suffering from chronic anemia due to repeated hemorrhages is associated with depletion of the constituents necessary for pigment production.

## EXPLANATION OF PLATES.

## PLATE 47.

FIG. 1. Humeri of Rabbits 1, 4, 6, and 18 at left—Nos. 5, 2, 7, and 15 at right (see Text-figs. 3 and 4). Those of the injected animals are on the left. The marrow in them is of dark red "currant jelly" character whereas that of the controls is for the most part fatty. Fresh specimens.

## PLATE 48.

FIG. 2. Femora of the same rabbits grouped in the same way and showing the same differences.

## PLATE 49.

FIGS. 3 and 4. These figures are taken from the same level in the distal portion of the femur marrow of Rabbits 11 and 13, an injected animal and its control which were bled almost equal amounts (Text-fig. 1). The replacement of fat tissue with hematopoietic elements is seen to be almost complete in Fig. 3, from the injected animal. The abundance and large size of the erythropoietic islands, as indicated by aggregations of pycnotic nuclei, are especially noteworthy.

592 .



592'

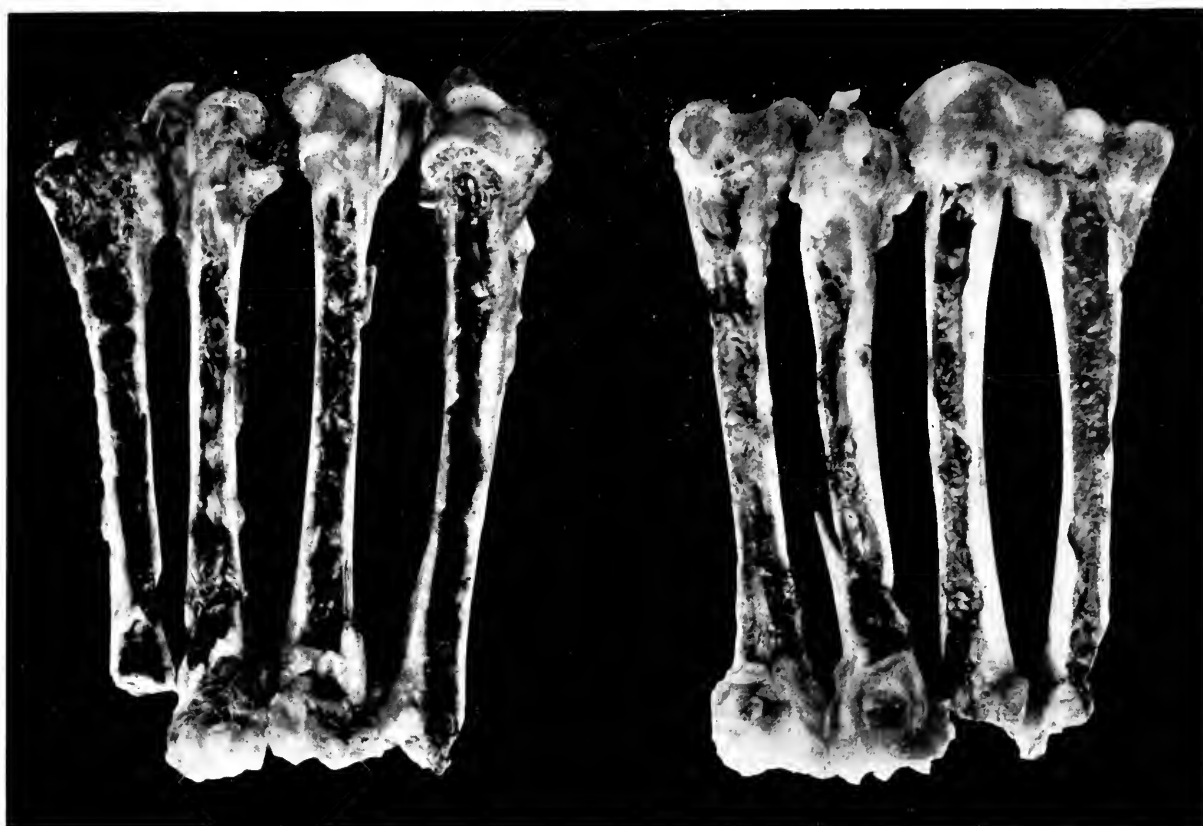


FIG. 1.

(McMaster and Haessler: Spread of red marrow during anemia.



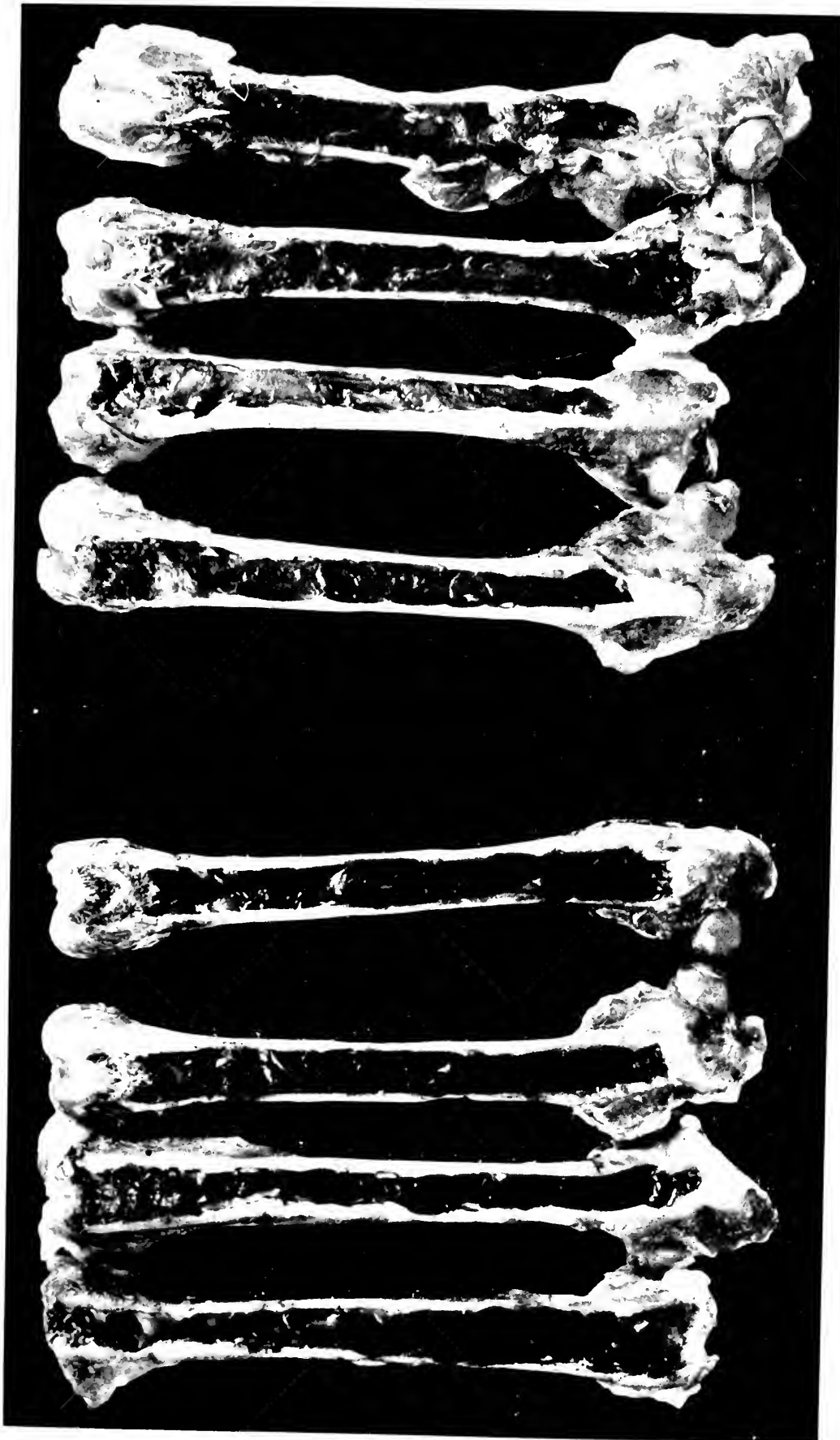


FIG. 2.

(McMaster and Haessler: Spread of red marrow during anemia.)



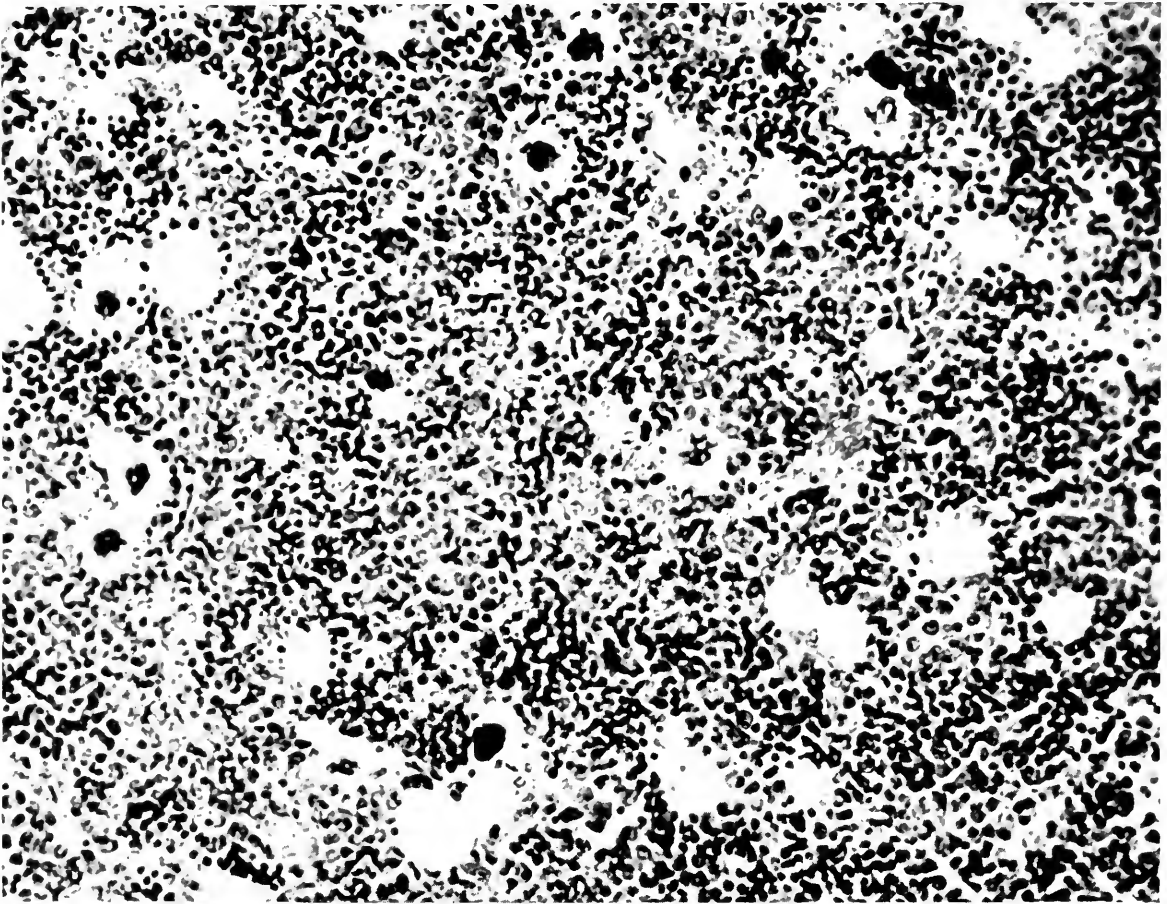


FIG. 3.

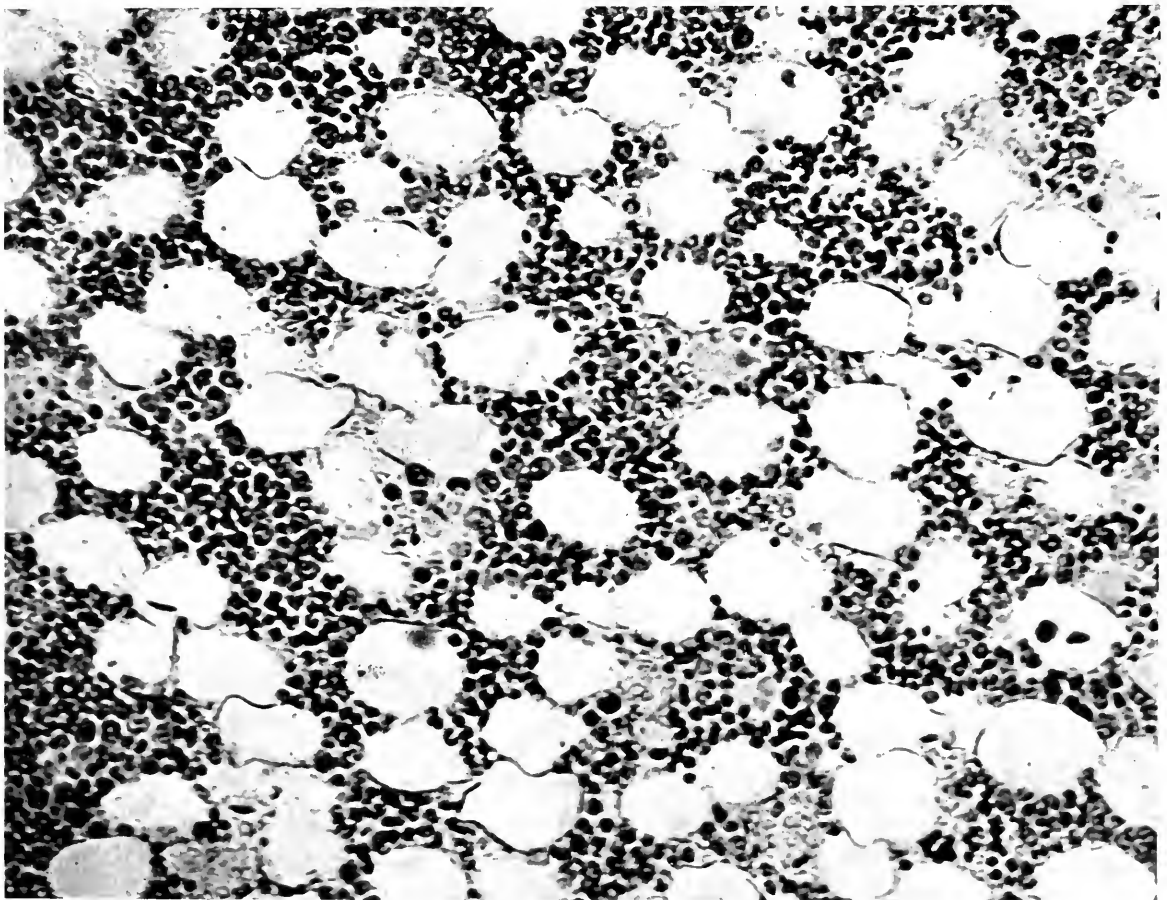


FIG. 4.

(McMaster and Haessler: Spread of red marrow during anemia.)



# THE CAPSULES OR SHEATHS OF *BACILLUS ACTINOIDES*.

By THEOBALD SMITH, M.D.

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PLATE 50.

(Received for publication, June 21, 1921.)

*Bacillus actinoides* is probably one member of a group of related bacteria, the other members of which will in due time be isolated and described. Its biological importance is defined by a production of abundant capsular substance on certain media<sup>1</sup> and by multiplication within epithelial cells,<sup>2</sup> its practical importance by its agency in the production of sporadic and epizootic bronchopneumonia in calves.<sup>2</sup>

In order to correlate the facts to be presented with what has been published the chief features of *Bacillus actinoides* are again briefly summarized.

1. Multiplication in the form of small flakes up to 1 mm. in diameter which consist of parallel bundles of sheathed filaments, each filament terminating in club-like expansions. The flake thereby becomes more or less rounded, mulberry-like, and resembles a sphere with clubs projecting from the central mass. Within the filaments of this *Actinomyces*-like growth are chains of minute bacilli.

2. The sheaths and clubs are produced in the condensation water of coagulated serum and less promptly on the sloped surface, but not on agar plus tissue, milk, or blood. On these latter media the growth is feeble and the organism appears in the form of rods. It often fails entirely.

3. On ordinary media, such as plain agar and bouillon, the bacillus multiplies very faintly or not at all.

4. After 10 or more days growth on agar plus blood or tissue, minute refringent bodies are found free in large numbers and also within the few remaining rods. In some cultures all bacilli had entirely disappeared. These statements apply to culture tubes made air-tight with sealing-wax.

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<sup>1</sup> Smith, T., *J. Exp. Med.*, 1918, xxviii, 333.

<sup>2</sup> Smith, T., *J. Exp. Med.*, 1921, xxxiii, 441.

5. The bacillus fails to produce any appreciable lesions in small animals but causes necrosis and suppuration both in the lungs and subcutis of calves. Its virulence gradually declines. In a strain between 3 and 4 years old it was practically extinct.

6. It is relatively vulnerable to destructive agents. It does not survive more than 2 or 3 weeks in sealed cultures. It is quite rapidly killed in dried films and in fluids at 60°C.

### *Cultural Characters.*

In the former paper, little was stated of the growth on the slanted surface of serum tubes, because the multiplication in the condensation water is prompt and striking, that on the surface slow and inconspicuous. Within 3 or 4 days the minute floccules appear in the relatively clear condensation water and within a week this fluid is opaque with them. On the sloped surface, however, mere point-like colonies, easily overlooked at first, do not appear for 6 or 7 days. If such tubes are kept thereafter at room temperature, the growth on the slanted surface slowly increases until in a month or 6 weeks colonies up to 0.5 mm. are present. They then appear as chalky-white specks, standing well up from the surface. Fusion into a flat, white, elevated patch is occasionally seen. The growth consists of a semifluid, opaque mass which microscopically is made up of the same elements as those found earlier in the condensation water.

The growth of *Bacillus actinoides* is much the same on coagulated cow serum and rabbit serum as on horse serum. The only points of difference are a slightly more vigorous growth on the cow serum and a slowly developing, distinct, pale yellowish tinge of the creamy floating layer and of the surface colonies.

Since the pneumonic lungs especially in later stages contain other species of bacteria, most frequently *Bacillus pyogenes* or *Bacillus bovisepiticus* or both, the detection of *Bacillus actinoides* may fail. The best medium to use in such cases is coagulated blood serum to which some calf serum water is added to increase the amount of "condensation water." This, however, is not essential. Even on the serum the secondary invaders grow with greater rapidity. Colonies of *Bacillus bovisepiticus* expand into very thin layers and those of *Bacillus pyogenes* pit the surface and cause slow liquefac-



tion. In such mixed cultures, *Bacillus actinoides* may be detected after a week as minute points between the other colonies. Microscopic examination shows the characteristic flocculi and bizarre forms associated with this species. It is frequently impossible, however, to obtain pure cultures from such tubes.

#### *Variations in the Production of Capsules.*

The absence of sheaths or capsules in cultures on agar slants containing blood or tissue, and on agar in which a small amount of milk with or without fat has been incorporated, has been referred to in part heretofore. On these surfaces the growth continues very feeble even after many transfers.

Absence of capsular substance has also been noticed on coagulated blood serum. From two cases both the sheathed and the unsheathed variety have been isolated and continued in pure culture indefinitely. When capsules are not produced the growth on the serum surface is very feeble and the chalky whiteness is replaced by a relatively colorless semitranslucent appearance of the point-like colonies. In the condensation water the floccules are replaced by a fine powdery cloudiness which is due to small groups and clusters of bacilli. Evidently the relative richness of the growth of the capsulated variety is chiefly due to the capsular material itself. Attention is called to this variety since it might be either overlooked on account of the very feeble growth or else regarded as some other species.

The same variations have appeared in the course of passing a given strain (No. 462) through calves. Both the sheathed and the non-sheathed variety appeared in cultures from the fourth calf. To determine whether the second form was virulent a calf was inoculated and a large local necrotic focus produced with it. The capsules are not therefore the carriers of virulence.

#### *Changes in the Sheaths or Capsules.*

Two modifications may take place, one occurring as the individual culture ages, the other after repeated transfers.

Concerning the first change little need be said. The sheaths in the early days of the culture are very feebly refringent. The floccules

dried on cover-slips appear as unstained spots with the tangle of stained bacilli within them. After a week or longer, they become more opaque, as if material were deposited within them. They then tend to absorb and hold dyes and the bacilli within them are no longer demonstrable.

The second change is one which progresses parallel with artificial cultivation. It may appear within one or several months. It consists in a gradual change in the physical appearance of the capsular material. When freshly isolated from the lungs, the sheath of *Bacillus actinoides*, examined unstained in the condensation water, suggests in its physical appearance the myelinic forms found in sputum and in nerve tissue (Fig. 1). After 1 or more months of weekly transfers, dense masses of very minute refringent granules, the largest about 0.5 micron in diameter, appear, which cover and conceal the floccules entirely (Fig. 3). These granules occur embedded in the sheath of the bacilli (Fig. 2). This transformation has taken place in all but one of the strains isolated. In this one (Calf 544) the absence of the particular change described even after 6 months of weekly transfers has<sup>1</sup> influenced the appearance of the culture. The mass and color of growth resemble closely the non-capsuled varieties described above. The growth of this strain on blood agar differs nowise from that of the capsulated strains.

#### *The Nature of the Sheath.*

The tendency of the growth mass in the condensation water of coagulated horse serum to form a creamy layer, floating at the surface, and a whitish deposit settling to the bottom, called attention to the possible lipoid character of the granules, since the creamy layer appears in quantity with the granules. When the tube is shaken this layer promptly forms a homogeneous suspension in the condensation water. After a day or two it reappears. This tendency to rise is not lost when the fluid containing the growth is heated up to 65°C.

The fine granules tend to run together into larger spheres when opportunity is given. If the surface growth spread on cover-glasses is allowed to dry for a few minutes and then examined in water,

larger refringent droplets or discs up to 6 or 8 microns in diameter have replaced most of the granules, owing to a coalescing of the latter into larger masses during the drying. The specimen then suggests a film of cream. It has a greasy appearance and acts as a greasy film in that air bubbles are usually imprisoned over it when water is placed on the slide and the cover-slip laid on it.

The dried film when placed in absolute alcohol, ether, chloroform, or petroleum ether is greatly altered. When the cover-slip is removed from these fluids, the adhering solvent permitted to evaporate, and the film examined in a drop of water, the refringent discs have disappeared. A slight amount of amorphous material which takes stains feebly and diffusely remains. Bacteria hitherto obscured by the mass of discs appear in large numbers even when unstained. After immersion in 5 per cent acetic acid and 5 per cent caustic potash little or no change in the fat-like bodies is seen. Scharlach R in alcoholic solution stains the fat-like droplets.

The above observations upon the presence of fat in the creamy layer have been confirmed by Paul E. Howe of this Department who has been examining the material as it became available from cultures, since no mass cultures have been attempted for a more detailed chemical analysis. The results of the preliminary chemical examination demonstrate the presence of from 50 to 66 per cent of fat or fat-like substance in the creamy layer which may be extracted from the dried material obtained by filtration of the culture fluid and subsequent drying at low temperature. From material separated and washed by suspension in one-half saturated sodium chloride 94 per cent of fat and fat-like substance was extracted. Certain types of crystals occurred in the extracted material, such as plates and feathery groups, which have not been identified as yet. Fatty acids were present in considerable amount and a sterol, probably cholesterol, has been demonstrated colorimetrically. However, a quantitative colorimetric estimation of the cholesterol present in the sterile culture fluid and of a similar fluid in which the organism had multiplied did not show any measurable difference in the amount of cholesterol present. A substance precipitated with acetone from ethereal solution has been repeatedly demonstrated. The quantities of material extracted have not exceeded 0.01 to 0.02 gm.

## SUMMARY.

The production of capsules or sheaths is subject to considerable variation in this species. Thus far coagulated serum has been found the only substrate to stimulate its appearance. It is not known whether the capsular substance is induced by the parasitism. The presence of sheaths within epithelial cells and their absence in cellular exudates suggest the inference that the sheath is a protective substance when the parasite multiplies in living cells. The complete absence of sheaths in some strains might make the recognition of this species difficult, but if its other characters both positive and negative are kept in mind, this difficulty will not be serious.

Next in importance to fluctuations in the presence or absence of capsules is the change in metabolic processes leading to the production and accumulation of fatty substances in the cultures. The original capsular material resembles in its optical characters myelins. In the course of artificial cultivation fat granules make their appearance at the expense of the original myelin-like substance. This process may be regarded as a degenerative one in the sense that it accompanies the gradual loss of the function of secreting the capsular substance.

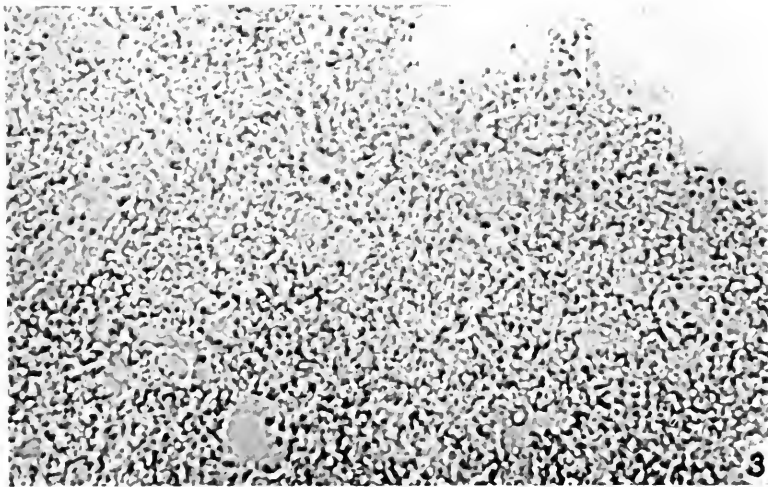
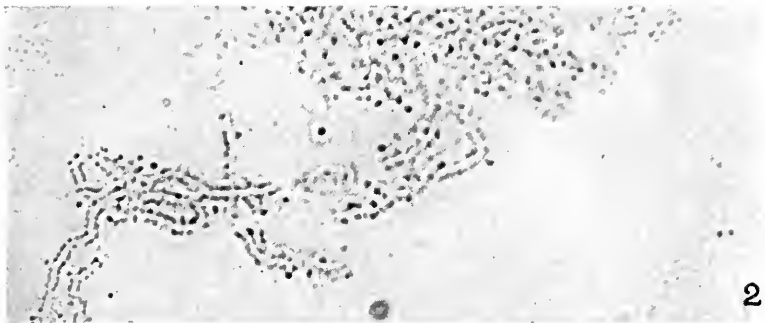
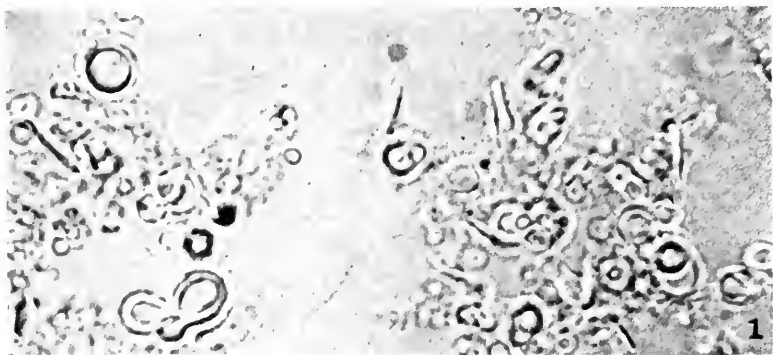
## EXPLANATION OF PLATE 50.

The preparations are unstained, mounted in normal salt solution (Fig. 1) and condensation water (Figs. 2 and 3).

FIG. 1. Horse serum culture of strain of *B. actinoides*, Calf 544. Growth from the sloped surface of a tube about 2½ months old, kept sealed at room temperature. Similar forms may be found in the condensation water of fresh cultures. This strain, under cultivation for 5 months, has thus far resisted the "fatty degeneration" tendency.  $\times 1,000$ .

FIG. 2. Condensation-water growth of a horse serum tube 2 days old. From strain of Calf 440, under cultivation for 18 months. Note the interlacing filaments with refringent granules embedded in the sheaths.  $\times 1,000$ .

FIG. 3. Similar growth from a culture, Calf 450, 2 days old. The strain has been under cultivation for 18 months. The figure represents a small portion of a floccule covered with refringent granules, among which are clear spaces representing the original myelin-like clubs and sheaths.  $\times 1,000$ .



(Smith: Capsules or sheaths of *Bacillus actinoides*.)



## AGE AND MULTIPLICATION OF FIBROBLASTS.

By ALEXIS CARREL, M.D., AND ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 11, 1921.)

The symptoms of senescence are evidently the expression of profound physicochemical changes which occur in the humors and the tissues of the organism under the influence of time. But the nature of these changes is still utterly unknown. If the modifications of blood serum and interstitial lymph which are functions of the age of the organism could be quantitatively studied, it is probable that the mechanism of growth, differentiation, and senescence would be better understood. Some years ago, one of the writers attempted to develop a method for detecting these modifications. By cultivating connective tissue in the plasma of chickens of different ages he found that the growth was more abundant in the plasma of the younger than in that of the older animals.<sup>1</sup> This fact suggested that proliferating fibroblasts could be used as a reagent for the changes occurring in the blood under the influence of age, if a technique sufficiently accurate were developed. During the last few years, the necessary improvements in the methods of cultivating tissues have been realized.<sup>2,3</sup> It has, then, become possible to study the cause of the slower multiplication of fibroblasts in the serum of old animals and the factors of senescence. The experiments of Loeb and Northrop on the temperature coefficient of duration of life of *Drosophila* lead to the conclusion that the duration of life was probably determined by the production of a substance leading to old age, or by the destruction of a substance which normally prevents old age and natural death.<sup>4</sup> It was important to know which of these

<sup>1</sup> Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

<sup>2</sup> Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

<sup>3</sup> Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

<sup>4</sup> Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

hypotheses agrees with the reality. The purpose of the experiments described in this paper was to ascertain whether a definite relation exists between the rate of multiplication of fibroblasts cultivated in plasma and the age of the animal from which the plasma is obtained, and whether the modifications brought about by age in the action of the plasma on fibroblasts are due to the disappearance of an accelerating factor, or to the production of an inhibiting factor.

## I.

*Relation of Age of Serum to Rate of Growth and Duration of Life of Fibroblasts.*

While it has been known for several years that the rate of growth of connective tissue varies in inverse ratio to the age of the animal from

TABLE I.

*Rate of Growth of Embryonic Heart and Liver in the Plasma of Chickens 2 Years, 5 Years, and 4 Months Old.*

Experiment No.	Culture No.	Date.	Nature of tissue.	Width of ring of new tissue.			Remarks.
				2 yr. old chicken plasma.	5 yr. old chicken plasma.	4 mo. old chicken plasma.	
		1912					
1	2321-1	Sept. 17	8 day embryo heart.	2.0		4.0	The plasma was taken from the animals, Sept. 17, 1912.
2	2321-2	" 17	8 " " "	1.5-2.0		3.5-4.0	
3	2321-3	" 17	8 " " "	1.5-2.0		3.5-4.0	
4	2326	" 18	7 " " "		1.5	3.0	
5	2335-1	" 20	7 " " "		2.0	3.0	
6	2335-2	" 20	7 " " liver.		1.2	2.5-3.0	
7	2335-3	" 20	7 " " "		1.0	2.5	
8	2342	" 21	7 " " heart.		2.0	3.0	
9	2356	" 23	7 " " "		2.0	2.5	

which the plasma is taken,<sup>1</sup> the relation between the two quantities could not be established, owing to the lack of sufficient accuracy of the technique. In 1912, fragments of embryonic heart and liver were cultivated in plasma obtained from 4 month, 2 year, and 5 year old chickens (Table I). The width of the ring of connective tissue pro-



duced around the fragments was larger in the plasma of the young than in that of the older chickens. A larger amount of connective tissue was produced in the serum of a 1 month old kitten than in that of a 9 year old cat. The same phenomenon occurred when the sera of 20 and 45 year old human beings were used as media. Con-

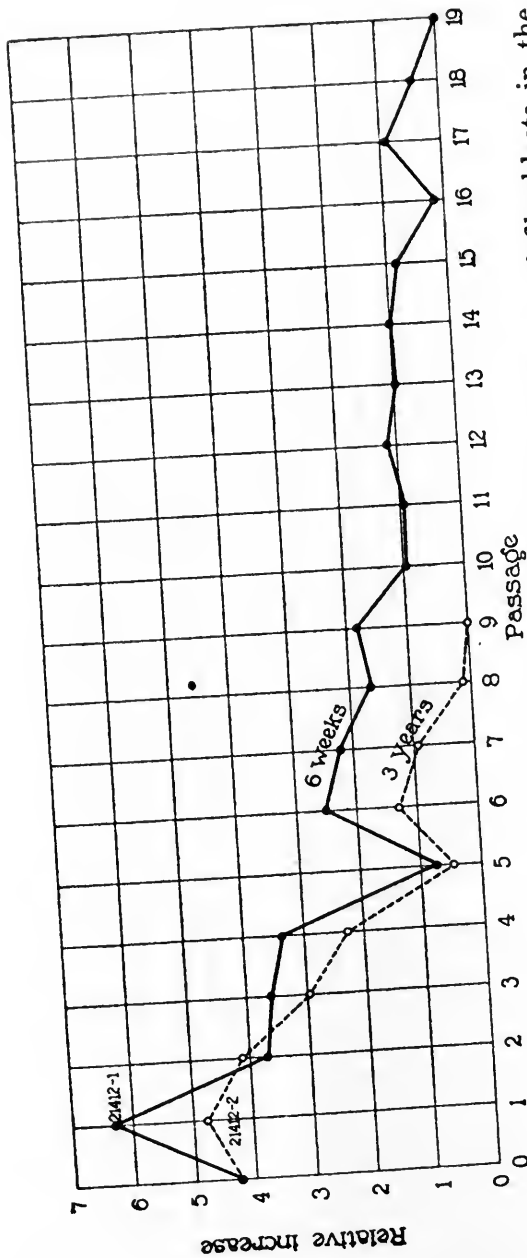
TABLE II.

*Rate of Growth and Duration of Life of Fibroblasts in the Plasma of a 6 Week Old Chicken and a 3 Year Old Hen.*

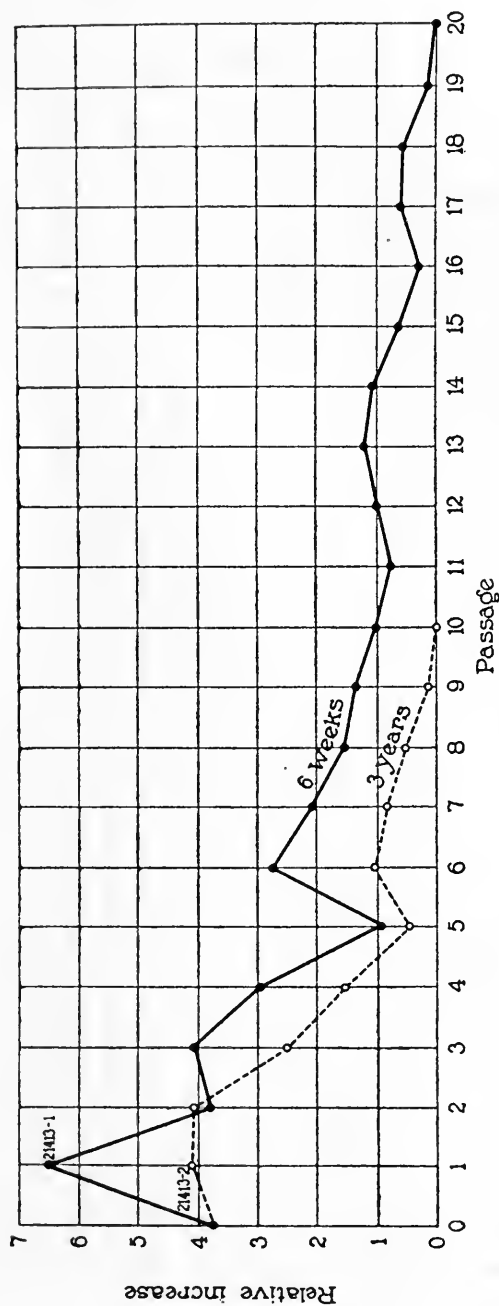
Experiment 1.					Experiment 2.					Experiment 3.				
Passage No.	Culture No.	Date.	Relative increase.		Passage No.	Culture No.	Date.	Relative increase.		Passage No.	Culture No.	Date.	Relative increase.	
			Young plasma.	Adult plasma.				Young plasma.	Adult plasma.				Young plasma.	Adult plasma.
		1921					1921					1921		
1	21412	May 12	6.3	4.78	1	21413	May 12	6.54	4.12	1	21414	May 12	6.06	3.86
2	21444	" 14	3.7	4.13	2	21446	" 14	3.8	4.09	2	21448	" 14	3.59	3.74
3	21485	" 16	3.61	2.97	3	21487	" 16	4.08	2.5	3	21489	" 16	4.47	2.85
4	21522	" 18	3.36	2.26	4	21524	" 18	2.96	1.55	4	21526	" 18	4.5	1.26
5	21548	" 20*	0.75	0.45	5	21550	" 20*	0.95	0.47	5	21552	" 20*	1.22	0.45
6	21582	" 21	2.52	1.31	6	21584	" 21	2.75	1.04	6	21586	" 21	2.37	1.22
7	21598	" 23	2.22	0.91	7	21600	" 23	2.08	0.85	7	21602	" 23	2.09	0.62
8	21636	" 25	1.64	0.13	8	21638	" 25	1.56	0.53	8	21640	" 25	1.96	0.42
9	21672	" 27	1.82	0	9	21674	" 27	1.35	0.21	9	21676	" 27	1.26	0.33
10	21714	" 29	0.97		10	21716	" 29	1.62	0	10	21718	" 29	1.09	0
11	21732	" 31	0.93		11	21733	" 31	0.76		11	21734	" 31	0.98	
12	21765	June 2	1.17		12	21766	June 2	1.0		12	21767	June 2	1.02	
13	21795	" 4	0.97		13	21796	" 4	1.21		13	21797	" 4	0.71	
14	21823	" 6	1.0		14	21824	" 6	1.08		14	21825	" 6	0.85	
15	21857	" 8	0.85		15	21858	" 8	0.61		15	21859	" 8	1.0	
16	21876	" 10	0.15		16	21877	" 10	0.27		16	21878	" 10	0.3	
17	21894	" 11	0.9		17	21895	" 11	0.60		17	21896	" 11	0.4	
18	21906	" 13	0.45		18	21907	" 13	0.36		18	21908	" 13	0.48	
19	21925	" 15	0		19	21926	" 15	0.15		19	21927	" 15	0.5	
					20	21947	" 17	0		20	21948	" 17†	0.74	
										21	21974	" 20	0.25	
										22	21992	" 22	0.26	

\* 24 hour culture.

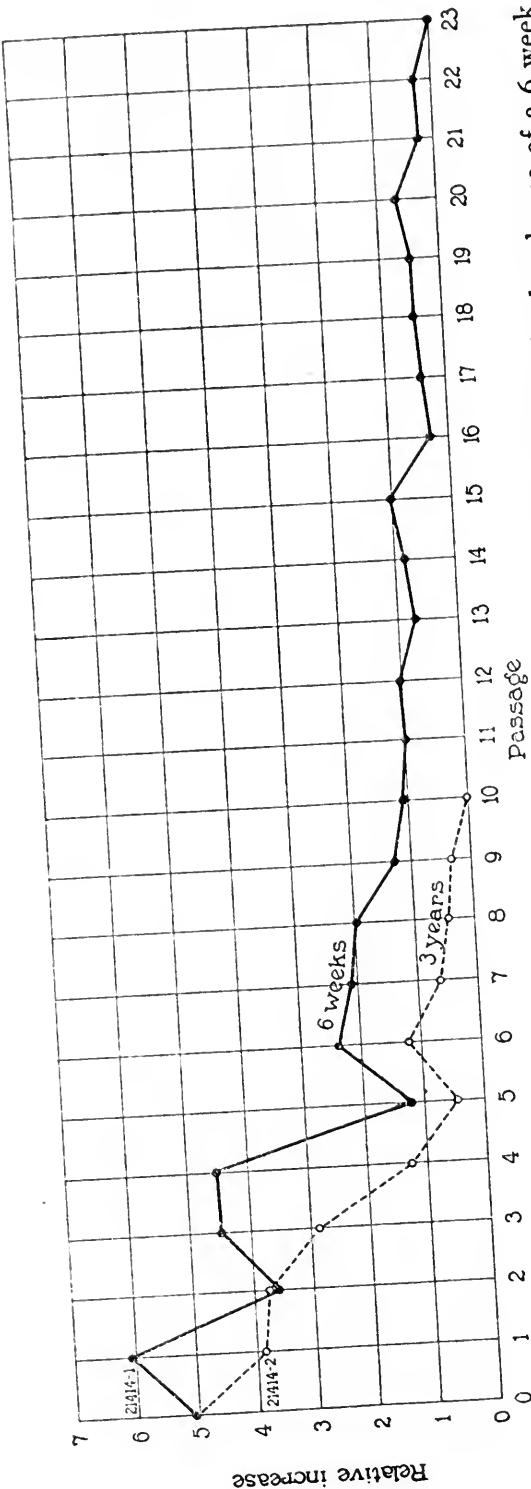
† 72 hour culture.



TEXT-FIG. 1. Experiment 1. Comparison of the rate of growth of fibroblasts in the plasma of a 6 week old chicken and a 3 year old hen. The sudden drop in the rate of growth of the culture in the 6 week plasma was accidental. The small amount of growth obtained at the fifth passage was due to the transfer of the tissues to a fresh medium after 24 hours instead of 48 hours.



TEXT-FIG. 2. Experiment 2. Comparison of the rate of growth of fibroblasts in the plasma of a 6 week old chicken and a 3 year old hen. The sudden drop in the rate of growth of the culture in the 6 week plasma was accidental. The small amount of growth obtained at the fifth passage was due to the transfer of the tissues to a fresh medium after 24 hours instead of 48 hours.



TEXT-FIG. 3. Experiment 3. Comparison of the rate of growth of fibroblasts in the plasma of a 6 week old chicken and a 3 year old hen. The sudden drop in the rate of growth of the culture in the 6 week plasma was accidental. The small amount of growth obtained at the fifth passage was due to the transfer of the tissues to a fresh medium after 24 hours instead of 48 hours.

nective tissue always grew more abundantly in the serum of the younger animals. In spite of the defects of the technique, the influence of age was evident.

Another series of experiments was made recently with more precise measurements. Pure cultures of fibroblasts were taken from a 9 year old strain of connective tissue previously kept in a mixture of plasma and embryo juice. Each fragment was divided in two equal parts which were imbedded in the plasma of chickens 6 weeks to 9 years of age. The proliferation of the fibroblasts in young and older plasmas was studied according to a technique previously described.<sup>3</sup> 1 hour after the preparation of the cultures, and 48 hours later, the fragments were outlined and their area was measured. The rate of growth was expressed in terms of the relative increase of the area of the original fragment in 48 hours. A study was made of the rate of growth and the duration of life of the fibroblasts in the plasma of 6 week, 3 month, 3 year, and 9 year old chickens.

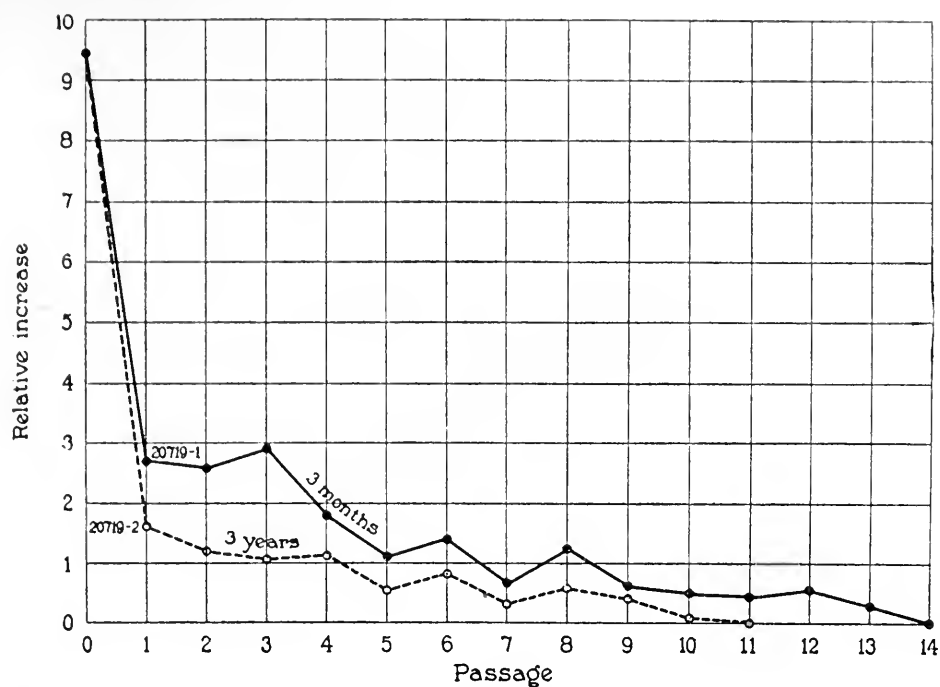
1. *Plasma of 6 Week Old and 3 Year Old Chickens.*—The plasma of animals 6 weeks and 3 years of age was used as a medium for fibroblasts taken from a 24 hour old stock culture. In every experiment, the growth was more active in the young than in the older plasma (Table II). On account of the accidental irregularities of the curves, the comparison of the rates of growth was made after the fifth passage (Text-figs. 1 to 3). The average amount of tissue produced during this period in the plasma of the older animal was 35 per cent of that in the plasma of the young animal. It was also found that the duration of life of the fibroblasts in the plasma of the older animal was 46 per cent of that in the plasma of the younger animal.

2. *Plasma of 3 Month Old and 3 Year Old Chickens.*—A comparison of the plasmas of 3 month and 3 year old animals was made in the same manner (Table III). The fibroblasts were taken from a 48 hour old stock culture. As soon as they were transferred from the mixture of plasma and embryo juice to plasma alone, a sudden drop in the velocity of growth was observed, as is shown by the descending branch of the curves (Text-figs. 4 to 7). This resulted from the suppression in the medium of the active substances contained in the

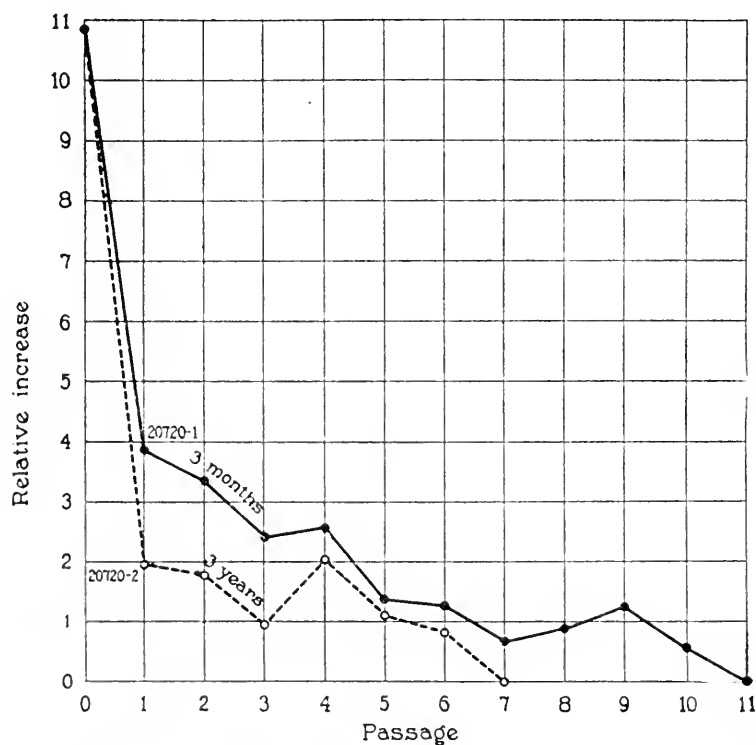
TABLE III.  
*Rate of Growth and Duration of Life of Fibroblasts in the Plasma of a 3 Month Old Chicken and a 3 Year Old Hen.*

Experiment 1.					Experiment 2.					Experiment 3.					Experiment 4.				
Passage No.	Culture No.	Date.	Relative increase.		Passage No.	Culture No.	Date.	Relative increase.		Passage No.	Culture No.	Date.	Passage No.	Culture No.	Date.	Relative increase.		Passage No.	Culture No.
			Young plasma.	Adult plasma.				Young plasma.	Adult plasma.							Young plasma.	Adult plasma.		
		1921					1921					1921			1921				
1	20719	Apr. 12	2.7	1.64	1	20720	Apr. 12	3.87	1.98	1	20721	Apr. 12	1	20722	Apr. 12	3.43	1.48	1	20722
2	20779	" 14	2.6	1.22	2	20781	" 14	3.36	1.78	2	20783	" 14	2	20785	" 14	2.73	1.81	2	20785
3	20846	" 16	2.9	1.06	3	20848	" 16	2.42	0.98	3	20850	" 16	3	20852	" 16	2.98	1.75	3	20852
4	20880	" 18	1.8	1.12	4	20882	" 18	2.57	2.03	4	20884	" 18	4	20886	" 18	2.13	1.17	4	20886
5	20928	" 20	1.1	0.53	5	20930	" 20	1.39	1.11	5	20932	" 20	5	20934	" 20	1.82	1.21	5	20934
6	20993	" 22	1.4	0.83	6	20995	" 22	1.27	0.81	6	20997	" 22	6	20999	" 22	1.63	0.78	6	20999
7	21067	" 25*	0.66	0.31	7	21069	" 25*	0.69	0	7	21071	" 25*	7	21073	" 25*	1.11	0	7	21073
8	21113	" 27	1.24	0.61	8	21115	" 27	0.88		8	21116	" 27	8	21117	" 27	0.97		8	21117
9	21156	" 29	0.65	0.4	9	21158	" 29	1.24		9	21159	" 29	9	21160	" 29	1.08		9	21160
10	21121	May 2*	0.5	0.09	10	21123	May 2*	0.58		10	21224	May 2*	10	21225	May 2*	0.39		10	21225
11	21259	" 4	0.44	0	11	21261	" 4	0		11	21262	" 4	11	21263	" 4	0.42		11	21263
12	21298	" 6	0.56							12	21299	" 6	12	21300	" 6	0.3		12	21300
13	21338	" 8	0.29							13	21339	" 8	13	21340	" 8	0.2		13	21340
14	21375	" 10	0							14	21376	" 10	14	21377	" 10	0		14	21377
													15	21408	" 12			15	21408

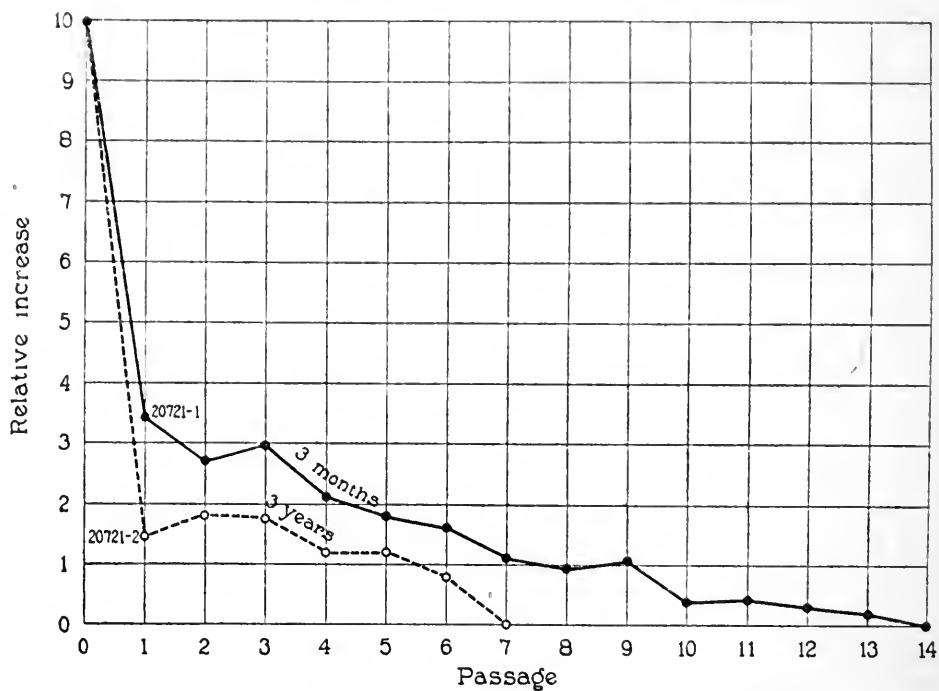
\* 72 hour culture.



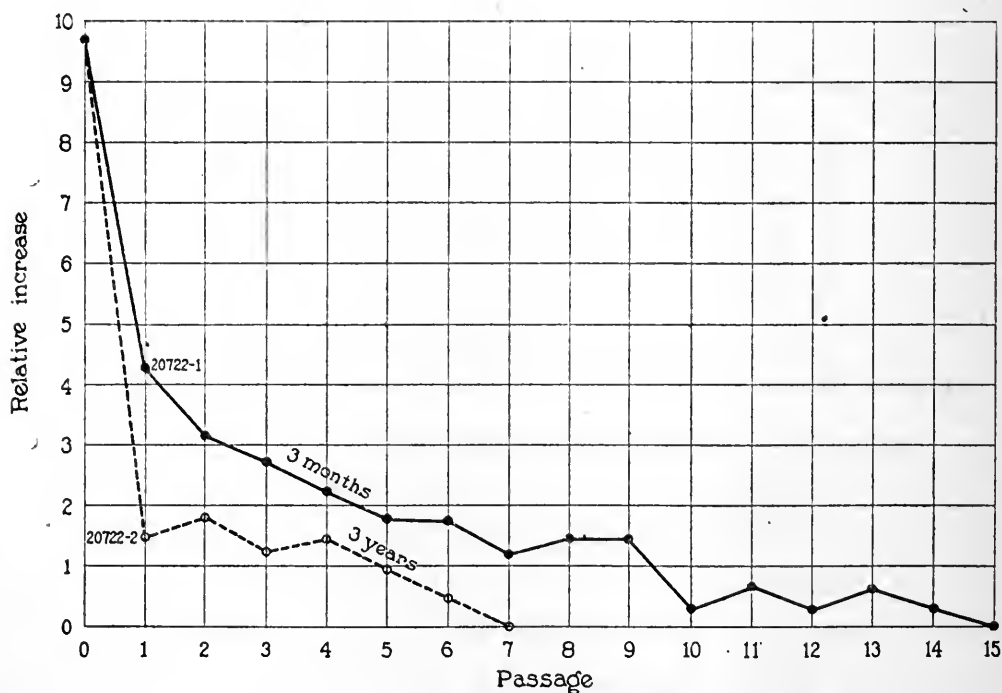
TEXT-FIG. 4. Experiment 1. Comparison of the rate of growth of fibroblasts in the plasma of a 3 month old chicken and a 3 year old hen.



TEXT-FIG. 5. Experiment 2. Comparison of the rate of growth of fibroblasts in the plasma of a 3 month old chicken and a 3 year old hen.



TEXT-FIG. 6. Experiment 3. Comparison of the rate of growth of fibroblasts in the plasma of a 3 month old chicken and a 3 year old hen.



TEXT-FIG. 7. Experiment 4. Comparison of the rate of growth of fibroblasts in the plasma of a 3 month old chicken and a 3 year old hen.



embryo juice.<sup>5,6</sup> After the first passage of both fragments in the plasma of the young and older chickens, a marked and almost con-

TABLE IV.

*Rate of Growth and Duration of Life of Fibroblasts in the Plasma of a 3 Month Old Chicken and a 9 Year Old Cock.*

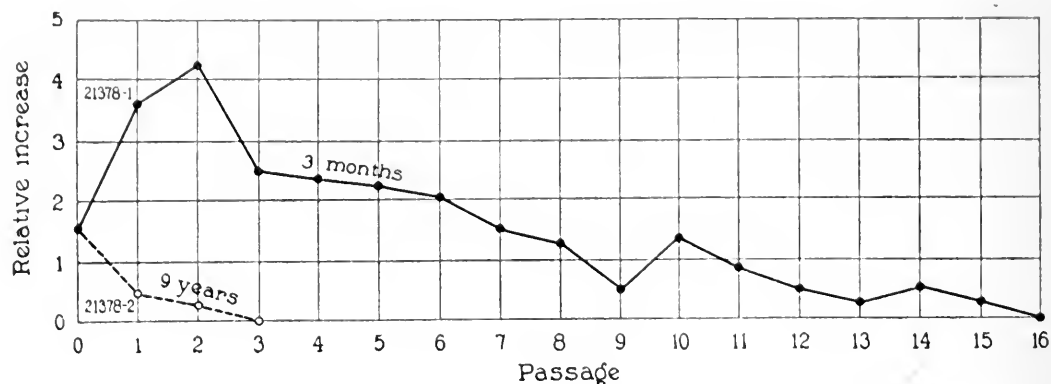
Experiment 1.					Experiment 2.					Experiment 3.				
Passage No.	Culture No.	Date.	Relative increase.		Passage No.	Culture No.	Date.	Relative increase.		Passage No.	Culture No.	Date.	Relative increase.	
			Young plasma.	Adult plasma.				Young plasma.	Adult plasma.				Young plasma.	Adult plasma.
		1921					1921					1921		
1	21244	May 3	3.65	0.48	1	21245	May 3	2.14	0.55	1	21246	May 3	4.56	2.08
2	21279	" 5	4.28	0.28	2	21281	" 5	3.62	0	2	21283	" 5	4.62	1.04
3	21318	" 7	2.52	0	3	21320	" 7	2.46		3	21321	" 7	3.17	0
4	21378	" 10	2.38		4	21379	" 10	1.84		4	21380	" 10	2.07	
5	21409	" 12	2.27		5	21410	" 12	2.15		5	21411	" 12	2.0	
6	21441	" 14	2.09		6	21442	" 14	1.58		6	21443	" 14	1.48	
7	21482	" 16	1.53		7	21483	" 16	1.50		7	21484	" 16	1.29	
8	21519	" 18	1.29		8	21520	" 18	1.56		8	21521	" 18	1.38	
9	21545	" 20*	0.55		9	21546	" 20*	0.61		9	21547	" 20*	0.49	
10	21579	" 21	1.34		10	21580	" 21	1.63		10	21581	" 21	2.63	
11	21595	" 23	0.83		11	21596	" 23	0.75		11	21597	" 23	0.49	
12	21633	" 25	0.50		12	21634	" 25	0.61		12	21635	" 25	0.25	
13	21669	" 27	0.26		13	21670	" 27	0.45		13	21671	" 27	0.17	
14	21711	" 29	0.52		14	21712	" 29	0.68		14	21713	" 29	0.3	
15	21729	" 31	0.26		15	21780	" 31	0.51		15	21731	" 31	0	
16	21763	June 2	0		16	21764	June 2	0						

\* 24 hour culture.

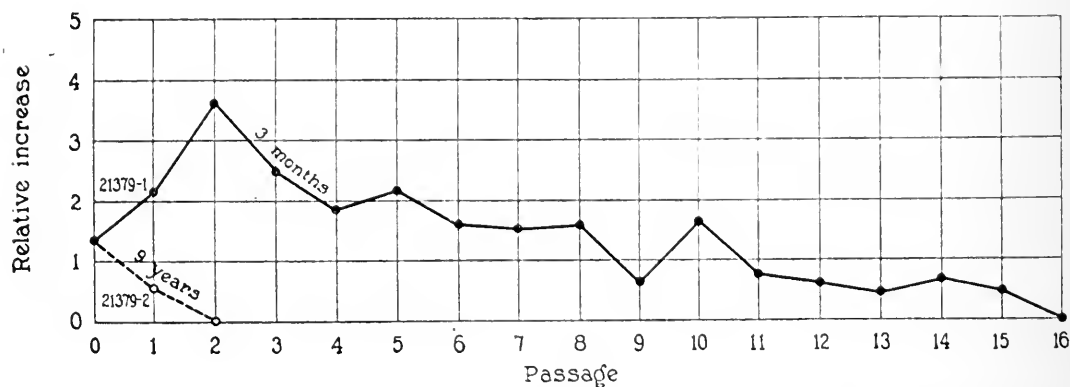
stant difference in the extent of growth was observed. The number of passages during which the fibroblasts multiplied, that is, the duration of the life of the cultures in both plasmas, differed widely. The tissues cultivated in the plasma of the older animal died after the seventh passage on an average, while in the plasma of the young

<sup>5</sup> Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

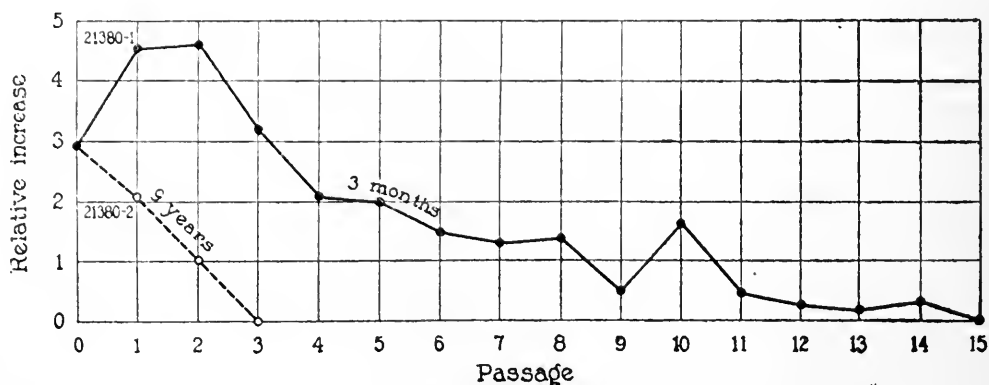
<sup>6</sup> Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.



TEXT-FIG. 8. Experiment 1. Comparison of the rate of growth of fibroblasts in the plasma of a 3 month old chicken and a 9 year old cock.

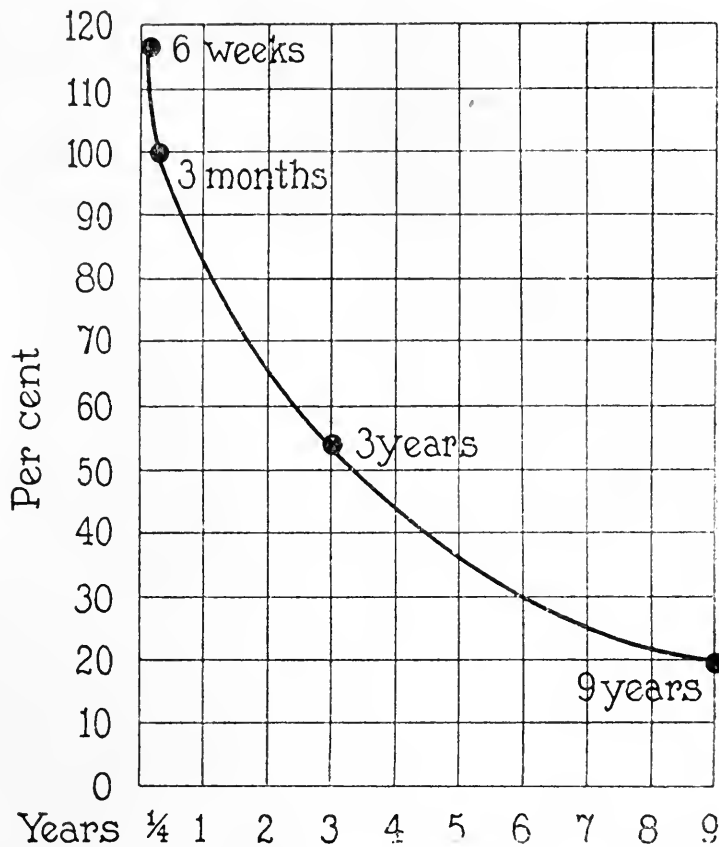


TEXT-FIG. 9. Experiment 2. Comparison of the rate of growth of fibroblasts in the plasma of a 3 month old chicken and a 9 year old cock.



TEXT-FIG. 10. Experiment 3. Comparison of the rate of growth of fibroblasts in the plasma of a 3 month old chicken and a 9 year old cock.

animal, death occurred after the thirteenth passage. Thus, the duration of life of connective tissue in the 3 year old plasma was 54 per cent of that in the 3 month old plasma (Text-fig. 11). The amount of tissue produced in a given time in both plasmas showed striking differences. A comparison was made between the areas of new



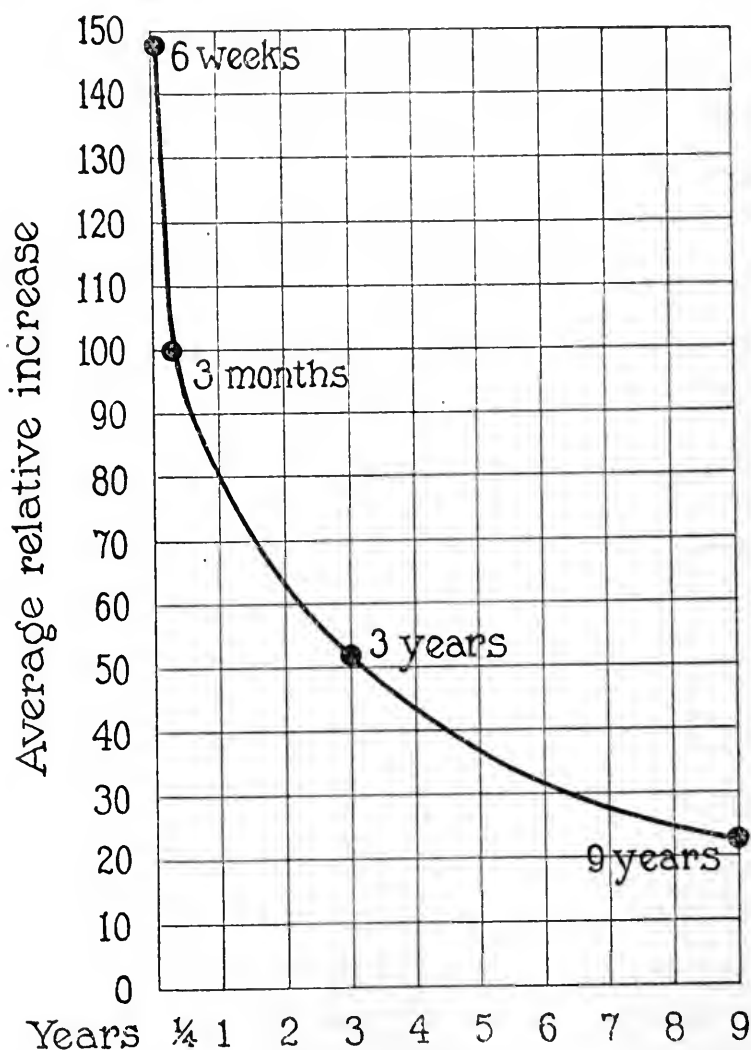
TEXT-FIG. 11. Duration of life *in vitro* of fibroblasts in function of the age of the animals from which the plasma was taken.

tissue produced in both plasmas during the life of the fibroblasts in the older plasma. The amount of growth in the plasma of the 3 year old animal was 52 per cent of that in the plasma of the 3 month old animal (Text-fig. 12).

3. *Plasma of 3 Month Old and 9 Year Old Chickens.*—The action of the plasma of a 3 month old chicken was compared with that of the plasma of a 9 year old chicken (Table IV, Text-figs. 8 to 10). The rate of growth in the old plasma was 23 per cent of that in the young

plasma (Text-fig. 12). The duration of life of the cultures in the plasma of the old animal was only 19 per cent of that in the plasma of the young animal (Text-fig. 11).

4. *Serum of 3 Month Old and 3 Year Old Chickens.*—An investiga-



TEXT-FIG. 12. Rate of growth of fibroblasts in function of the age of the animal from which the plasma was taken.

tion was then made in order to determine whether serum acted in the same manner as plasma. Fragments of a pure culture of fibroblasts were placed in media composed of fibrinogen 20 per cent, Tyrode solution 10 per cent, and serum 70 per cent (Table V). The sera were obtained from the plasmas of the 3 month and 3 year old

animals. It was soon found that the original fragment often contracted in the serum of the older animals, and no accurate measurement was possible. In several cases, however, the cultures remained normal. The rate of growth during the first passage in the serum of the 3 year old chicken was 69 per cent of that in the serum of the 3 month old chicken. Other experiments were made in which the fibrinogen suspension was replaced in the medium by 30 per cent normal plasma. Again similar changes in the rate of growth were observed.

TABLE V.

*Rate of Growth of Fibroblasts in the Serum of a 3 Month Old and a 3 Year Old Hen.*

Experiment No.	Culture No.	Date.	Medium.	
			Young.	Adult.
			Serum 70 per cent; fibrinogen 20 per cent.	Serum 70 per cent; fibrinogen 20 per cent.
		1921		
1	20839	Apr. 16	2.28	1.44
2	20840	" 16	2.65	1.74
3	20841	" 16	1.91	1.47
4	20842	" 16	3.06	2.14
5	20843	" 16	3.03	2.42
6	20844	" 16	2.34	1.55
7	20845	" 16	3.13	1.89
Average .....			2.63	1.81

There was, therefore, no doubt that the rate of multiplication of fibroblasts and the duration of their life *in vitro* varied in inverse ratio to the age of the animals from which the plasma or the serum was taken. The graph representing the rate of growth, in function of the age of the animals which have given the plasma, shows that the differences are very large (Text-fig. 12). Another graph expresses the relation between the duration of life and the age (Text-fig. 11). These curves show that there is a definite relation between the rate of proliferation of the fibroblasts, the length of their life *in vitro*, and the age of the animal. It is, therefore, possible to use a pure culture of

fibroblasts as a reagent for detecting some of the modifications occurring in blood serum under the influence of time.

## II.

### *Relation of Concentration of Serum to Rate of Growth.*

The action on the growth of fibroblasts of the serum taken from animals of advancing age could be explained by the disappearance from the blood of an accelerating factor, or the development in the blood of an inhibiting factor. If youth were supposed to be characterized by a factor present in the blood and activating the multiplication of fibroblasts, and senescence by a decrease in the power of this factor, connective tissue cells should proliferate more

TABLE VI.

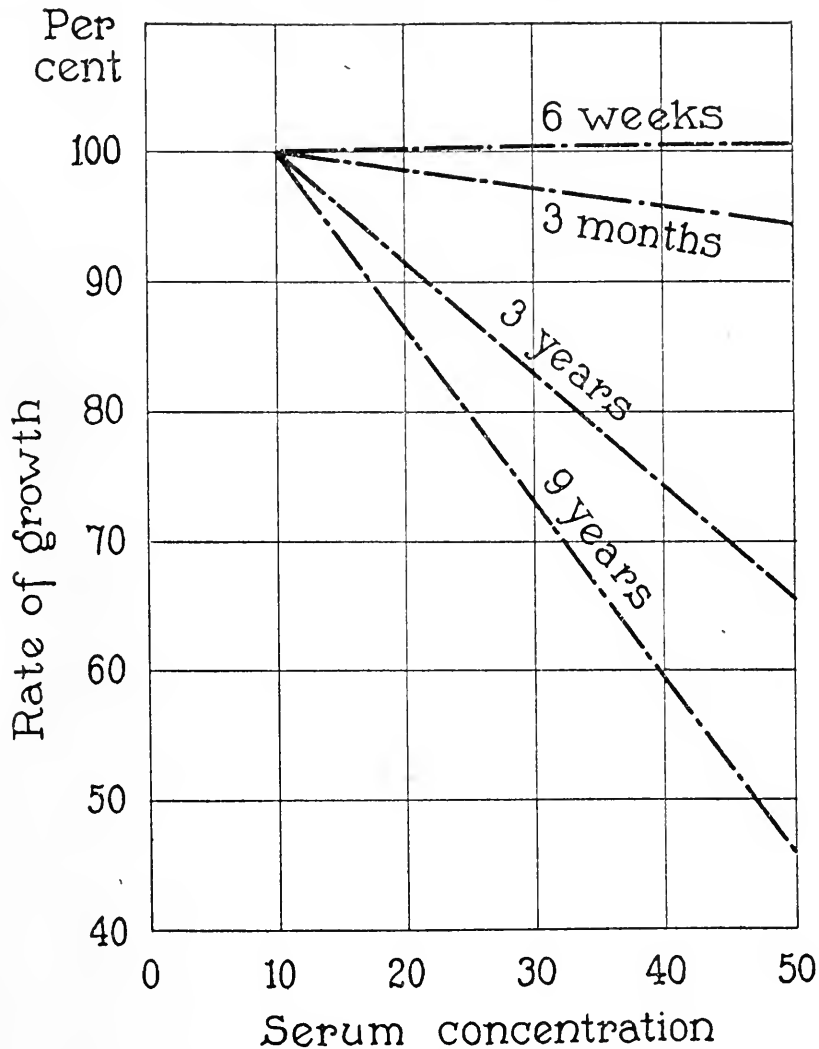
*Rate of Growth of Fibroblasts in High and Low Concentrations of the Serum of a 6 Week Old Chicken.*

Experiment No.	Culture No.	Date.	Medium.	
			Serum 10 per cent; fibrinogen 20 per cent.	Serum 50 per cent; fibrinogen 20 per cent.
		1921		
1	21424	May 13	4.34	3.90
2	21425	" 13	2.62	3.17
3	21426	" 13	3.74	4.21
4	21427	" 13	4.13	4.00
5	21428	" 13	3.58	3.35
Average.....			3.68	3.73

actively in a medium containing serum under a high concentration than in a medium made of a lower concentration of the same serum. On the contrary, if the same phenomena were caused by the progressive increase of a factor inhibiting the multiplication of fibroblasts, connective tissue cells should multiply more actively in a low than in a high serum concentration.

The value of these hypotheses was examined in a study of the growth of fibroblasts in high and low concentrations of the serum of

chickens 6 weeks, 3 months, 3 years, and 9 years of age. The media were made of fibrinogen suspension, serum, and Tyrode solution.



TEXT-FIG. 13. Variations of the rate of growth of fibroblasts in function of the serum concentration and of the age of the animal from which the serum was taken. The chart shows the modifications of the rate of growth in high and low serum concentrations of chickens 6 weeks, 3 months, 3 years, and 9 years old. The rate of growth in the lower serum concentration is considered equal to 100 per cent.

The serum concentrations used were generally 10, 50, and 70 per cent. When the serum of the older animal was used, the original fragment frequently contracted after a few hours. The growth was irregular and no accurate measurements could be taken. These changes

TABLE VII.

*Rate of Growth of Fibroblasts in High and Low Concentrations of the Serum of a 3 Month Old Chicken, and in Tyrode Solution.*

Experiment No.	Culture No.	Date.	Medium.		Medium.		Medium.		Medium.		Medium.	
			Serum 10 per cent; fibrinogen 20 per cent.	Serum 70 per cent; fibrinogen 20 per cent.	Serum 10 per cent; plasma 30 per cent.	Serum 70 per cent; plasma 30 per cent.	Tyrode solution 70 per cent; plasma 30 per cent.	Serum 70 per cent; plasma 30 per cent.	Serum 10 per cent; fibrinogen 20 per cent.	Serum 50 per cent; fibrinogen 20 per cent.	Serum 2.5 per cent; fibrinogen 20 per cent.	Serum 50 per cent; fibrinogen 20 per cent.
		1921										
1	20980	Apr. 21	3.89	4.04								
2	20981	" 21	2.79	2.66								
3	20982	" 21	3.05	3.55								
4*	21076	" 25			2.46	Shrunk-en.						
5	21077	" 25			2.58	2.25						
6	21078	" 25			4.55	4.31						
7*	21079	" 25					3.68	Shrunk-en.				
8	21080	" 25					2.32	1.02				
9	21081	" 25					4.0	2.81				
10	21100	" 26			3.70	2.57						
11	21101	" 26			3.40	2.05						
12	21102	" 26					3.70	2.57				
13	21103	" 26					3.40	2.05				
14	21167	" 29			4.50	3.47						
15	21168	" 29			3.65	2.84						
16	21169	" 29			4.37	4.33						
17	21170	" 29					6.43	2.64				
18	21171	" 29					3.05	3.20				
19	21172	" 29					3.70	1.40				
20	21227	May 2							1.28	1.33		
21	21228	" 2							1.09	1.03		
22	21229	" 2							1.62	1.26		
23	21230	" 2									1.79	1.74
24	21231	" 2									2.38	2.15
25	21232	" 2									1.73	1.93
26	21604	" 23							2.59	2.47		
27	21605	" 23							3.22	3.13		
28	21606	" 23							3.62	3.23		
29	21607	" 23							3.31	3.35		
Average.....			3.24	3.42	3.82	3.12	3.80	2.24	2.39	2.26	1.97	1.94

\* Not included in average.



occurred earlier and were more marked when the serum of the 9 year old chicken was used. This peculiar action of the serum of the older animals rendered necessary the use of a serum concentration of 50 per cent. In other experiments 30 per cent plasma was substituted for 20 per cent fibrinogen suspension. The action of serum was also compared with that of Tyrode solution alone.

1. *Serum of 6 Week Old Chicken.*—The media contained respectively 10 and 50 per cent serum, and 20 per cent fibrinogen suspension. Five experiments were performed (Table VI). The differences in the rate of growth in the low and high serum concentrations were practically the same (Text-fig. 13). Therefore, the serum of the 6 week old chicken could be considered as deprived of a retarding as well as of an accelerating action on connective tissue cell proliferation.

2. *Serum of 3 Month Old Chicken.*—The media contained 2.5, 10, 50, and 70 per cent serum, and was completed in some experiments by 20 per cent fibrinogen suspension, and in others by 30 per cent plasma. There was no marked difference in the rate of growth in the low and high concentrations of serum (Table VII). It seemed, therefore, that the serum of a 3 month old chicken did not contain any accelerating factor. The slight retardation of the rate of growth observed in the higher serum concentrations was too small to allow any conclusion about the presence or absence of an inhibiting factor (Text-fig. 13). In a last series of experiments, the differences in the rate of multiplication of fibroblasts produced by 0 and 70 per cent serum were examined. The amount of growth obtained in 70 per cent serum was only 58 per cent of that observed in Tyrode solution.

3. *Serum of 3 Year Old Chicken.*—In seven experiments, the growth of fibroblasts in fibrinogen medium containing 10 and 50 per cent serum was studied. In other experiments, 30 per cent plasma was substituted for fibrinogen suspension. The growth was found to be constantly smaller in the media containing 50 per cent serum than in that containing only 10 per cent (Table VIII). In the media made of fibrinogen and serum, the amount of tissue produced in the 50 per cent serum was only 66 per cent of the amount in 10 per cent serum (Text-fig. 13). Since an increase of 40 per cent in the concentration of the serum in the culture medium decreased the amount of tissue produced by 34 per cent, the serum of the 3 year old chicken con-

tained, without any doubt, a factor which inhibited markedly the proliferation of fibroblasts *in vitro*.

TABLE VIII.

*Rate of Growth of Fibroblasts in High and Low Concentrations of the Serum of a 3 Year Old Hen, and in Tyrode Solution.*

Experiment No.	Culture No.	Date.	Medium.		Medium.		Medium.	
			Serum 10 percent; fibrinogen 20 percent.	Serum 50 percent; fibrinogen 20 percent.	Serum 10 percent; plasma 30 percent.	Serum 70 percent; plasma 30 percent.	Tyrode solution 70 per cent; plasma 30 percent.	Serum 70 percent; plasma 30 percent.
		1921						
1	21001	Apr. 22			5.32	3.25		
2	21002	" 22			3.64	3.21		
3	21003	" 22			6.0	3.53		
4	21041	" 23			3.08	2.33		
5	21042	" 23			3.91	2.22		
6	21043	" 23			3.29	2.78		
7	21118	" 27			1.83	1.40		
8	21119	" 27			2.06	1.24		
9	21120	" 27			Spoiled.			
10	21121	" 27					3.13	1.48
11	21122	" 27					Spoiled.	
12	21123	" 27					2.18	1.24
13	21143	" 28			4.34	3.55		
14	21144	" 28			4.13	3.69		
15	21145	" 28			5.90	5.50		
16	21146	" 28			5.62	5.22		
17	21147	" 28			4.09	2.78		
18	21148	" 28			5.08	4.73		
19	21264	May 4	4.33	2.60				
20	21265	" 4	3.25	1.54				
21	21266	" 4	2.68	1.48				
22	21554	" 20	2.65	2.19				
23	21555	" 20	3.42	2.28				
24	21556	" 20	3.06	2.35				
25	21557	" 20	3.54	2.81				
Average.....			3.27	2.18	4.16	3.24	2.65	1.36

In a few experiments, the action of 70 per cent serum was compared with that of pure Tyrode solution, and under these conditions the serum also appeared to have a marked retarding action (Table VIII).

4. *Serum of 9 Year Old Chicken.*—In several experiments, no growth could be observed in the media containing 50 per cent serum because the tissue fragment contracted. However, in a few, the cultures remained normal and accurate measurements could be made (Table IX). The rate of growth in the high serum concentra-

TABLE IX.

*Rate of Growth of Fibroblasts in High and Low Concentrations of the Serum of a 9 Year Old Cock.*

Experiment No.	Culture No.	Date.	Medium.	
			Serum 10 per cent; fibrinogen 20 per cent.	Serum 50 per cent; fibrinogen 20 per cent.
		1921		
1*	21267	May 4	2.53	0
2*	21268	" 4	2.36	0
3*	21269	" 4	2.74	0
4	21304	" 6	1.82	1.03
5	21305	" 6	1.11	0.20
6	21306	" 6	1.60	0.80
7	21346	" 8	2.43	1.57
8	21347	" 8	3.24	1.46
9	21348	" 8	2.36	0.87
Average.....			2.09	0.99

\* Not included in average.

tion was only 46 per cent of that observed in the low serum concentration (Text-fig. 13). When the medium contained 70 per cent serum, the tissue fragment practically always contracted and showed very little activity. The cells contained more fat granules in the higher concentrations of serum than in the lower. It appeared that the serum of an old animal determined certain changes in the tissue which could not be detected by mere measurement of the rate of proliferation.

The results obtained in the preceding experiments are represented in Text-fig. 13, in which the amount of growth in the high serum concentration is compared with that in the lower, which is considered equal to 100 per cent. In no case did the fibroblasts multiply

more actively in high than in low serum concentration. This demonstrated that in growing animals, the blood serum does not contain any accelerating factor for the proliferation of the fibroblasts. The higher concentrations of the serum of the older animals retarded the rate of multiplication markedly. There was no doubt, therefore, that age does not bring about the disappearance from the blood serum of an accelerating factor, but produces the increase of an inhibiting factor for the growth of fibroblasts.

### III.

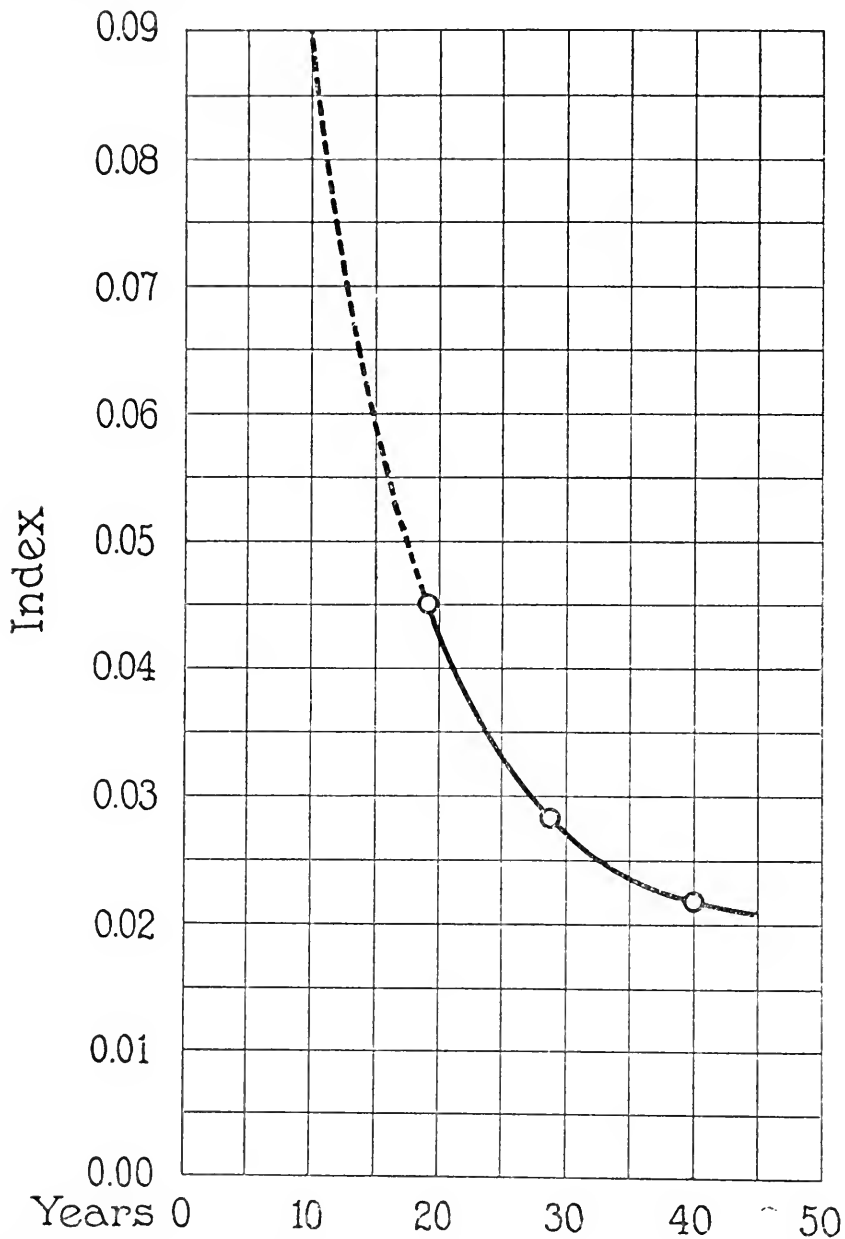
#### SUMMARY.

Pure cultures of fibroblasts displayed marked differences in their activity in the plasma of young, middle aged, and old chickens. The rate of cell multiplication varied in inverse ratio to the age of the animal from which the plasma was taken. There was a definite relation between the age of the animal and the amount of new tissue produced in its plasma in a given time (Text-figs. 1 to 10). The chart obtained by plotting the rate of cell proliferation in ordinates, and the age of the animal in abscissæ, showed that the rate of growth decreased more quickly than the age increased (Text-fig. 12). The decrease in the rate of growth was 50 per cent during the first 3 years of life, while in the following 6 years it was only 30 per cent. When the duration of the life of the cultures in the four plasmas was compared, a curve was obtained which showed about the same characteristics (Text-fig. 11). The duration of life of the fibroblasts *in vitro* varied in inverse ratio to the age of the animal, and decreased more quickly than the age increased.

As the differences in the amount of new tissue produced in the plasma of young, middle aged, and old chickens were large, the growth of a pure culture of fibroblasts could be employed as a reagent for detecting certain changes occurring in the plasma under the influence of age. But the method possesses the necessary accuracy only when it is used as has already been described,<sup>3</sup> and by technicians thoroughly trained in the details of its application.

A comparative study of the growth of fibroblasts in media containing no serum, and serum under low and high concentrations, was

made in order to ascertain whether the decreasing rate of cell multiplication was due to the loss of an accelerating factor, or to the in-



TEXT-FIG. 14. Curve showing the variations of the index of cicatrization of a wound 40 sq. cm. in area, in function of the age of the patient.

crease of an inhibiting one. In high and low concentrations of the serum of young animals, no difference in the rate of multiplication of fibroblasts was observed. This showed that the serum of an actively

growing animal did not contain any accelerating agent. The same experiments were repeated with the serum of a 3 year old and a 9 year old chicken. The medium made of a high concentration of serum had a markedly depressing effect on the growth, and this effect was greater in the serum of the older animal (Text-fig. 13).

"no chicken"

The results of the experiments showed in a very definite manner that certain changes occurring in the serum during the course of life can be detected by modifications in the rate of growth of pure cultures of fibroblasts, and that these changes are characterized by the increase of an inhibiting factor, and not by the loss of an accelerating one. It appeared, therefore, that the substances which greatly accelerate the multiplication of fibroblasts and are found in the tissues<sup>6</sup> do not exist in the blood serum, or are constantly shielded by more active inhibiting factors. The curve which expresses the variations of the inhibiting factor in function of the age was compared with that showing the variations of the rate of healing of a wound according to the age of the subject. For wounds of equal size, the index of cicatrization, which expresses the rate of healing, varies in inverse ratio to the age.<sup>7</sup> The different values of the index of cicatrization of a wound 40 sq. cm. in area, taken from measurements made by du Noüy,<sup>8</sup> were plotted in ordinates, and the age of the subject in abscissæ (Text-fig. 14). The curve showed a decrease in the activity of cicatrization which resembled the decrease in the rate of growth of fibroblasts in function of the age of the animal. This suggested the existence of a relation between the factors determining both phenomena.

#### IV.

#### CONCLUSIONS.

1. Under the conditions of the experiments and within the limits of accuracy of the method, there is a definite relation between the rate of growth of a pure culture of fibroblasts, cultivated in plasma, and the age of the animal from which the plasma is

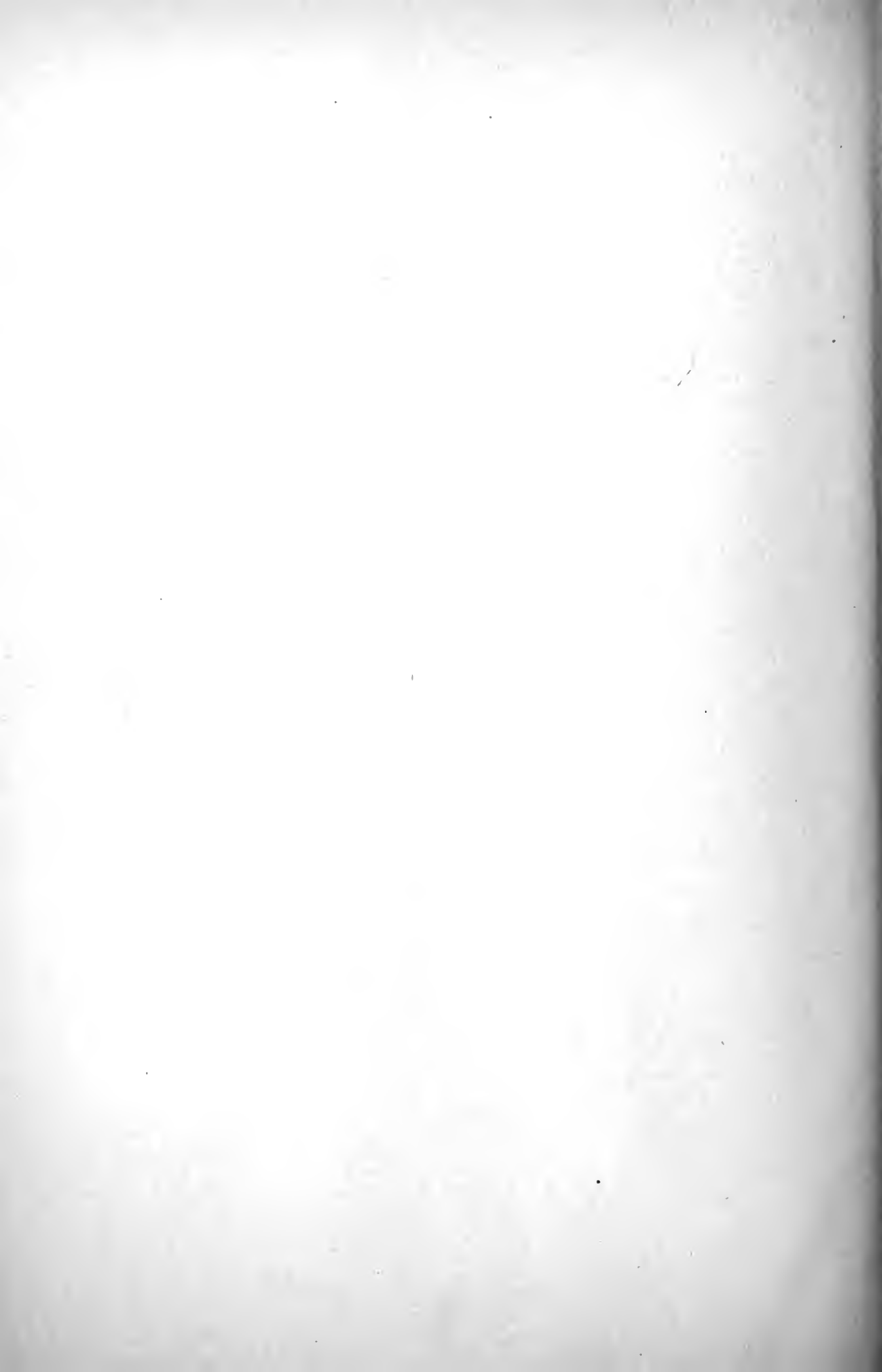
<sup>7</sup> du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451.

<sup>8</sup> du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 463.

obtained, the rate of cell multiplication varying in inverse ratio to the age. A similar relation exists between the duration of life *in vitro* of the fibroblasts, and the age of the animal.

2. The variations in the rate of growth of a pure culture of fibroblasts may be used as a reagent of certain modifications occurring in blood serum under the influence of age.

3. The action of age on serum is characterized, not by the decrease of an accelerating factor for the multiplication of fibroblasts, but by the increase of an inhibiting factor.





## STUDIES ON ENDOTHELIAL REACTIONS.

### V. THE ENDOTHELIUM IN THE HEALING OF ASEPTIC WOUNDS IN THE OMENTUM OF RABBITS.

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PLATES 51 TO 54.

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A study of the reaction of the capillary endothelium to various pathological conditions would be incomplete if the part played by its cells in aseptic wound healing were not considered. The fact that fibrils were demonstrated in the epithelioid cells in tuberculosis (Foot, 1921) makes it imperative to ascertain whether cells of like origin take part in the formation of fibers in the process of simple tissue repair after aseptic injury. As stated in previous papers in this series of studies on endothelial reactions (Foot, 1919, 1920, *a*, *b*, 1921), it may be assumed that the endotheliocyte<sup>1</sup> after leaving the vascular endothelium, may be transformed into larger forms, known as macrophages and by many other names. They may also fuse with like cells to form syncytia, or giant cells. What is their function in the formation of granulation tissue? We have seen that they may be vitally stained *in situ* by means of a 50 per cent mixture of Higgins' waterproof drawing ink and distilled water, administered intravenously; under these conditions they may be followed after they leave the parent tissue and migrate to other parts. Thereafter any cell in the omental lesion found to contain these ink globules is

<sup>1</sup> The name endotheliocyte has recently been applied to the small mononuclear endothelial cell, in contradistinction to the macrophage, or histiocyte, the larger form of this cell. It will be employed throughout this paper in connection with the endothelial cell in its small form, as seen shortly after emerging from the vessels.

presumably of endothelial origin, irrespective of its position in the tissue.

It must be borne in mind that this appears to be the only reliable means for identifying the endothelial cell *extra situ*. The use of the more diffusible colloidal stains, particularly those of the benzidine type, is not alone sufficient; they usually fail to stain the endothelium *in situ*, although they appear in its derivatives in the tissues outside of the vessels and also stain cells that were never a part of the endothelium. They are, therefore, only reliable as adjuncts to the colloidal carbon used in this connection. Recent articles, quoting this series of papers, seem to have missed this point; unless colloidal carbon be employed, accurate comparisons cannot be drawn. Aside from the liver epithelium, in which this pigment can be found after forced, prolonged administration, it is taken up only by cells which conceivably could have originated in the capillary endothelium. It is probably not phagocytosed by cells outside of the vessels, since it is never found escaping through their walls in a free state, unless there be hemorrhage; since it does not appear to be transferred from cell to cell, except when dead phagocytes are, in turn, phagocytosed by younger cells of similar origin; and since it apparently disappears from the circulating blood in a short time after its administration, being withdrawn by the endothelium of the liver, lungs, and bone marrow, or lying in the splenic sinuses or their endothelium. This localizes the carbon granules principally in the endothelium. Furthermore, the endothelium of peripheral capillaries in the connective tissues does not seem to take up the ink readily unless it is somewhat inflamed and swollen; this accounts for the fact that ink is so sparingly found in this situation in the earlier lesions of the series to be described later. When injected directly into the subcutaneous tissue, the carbon particles are taken up almost entirely by phagocytes and not by the sessile cells; fibroblasts, when part of the fixed tissues, seem to have so limited a capacity for incorporating these carbon granules, even when coated with them, that they may be considered as having no phagocytic function.<sup>2</sup>

<sup>2</sup>It seems probable that foreign particles are phagocytosed by these cells while they are wandering about and retained by them after they become sessile; the reasons for this assumption will be made evident further on in this paper.

Another point that should be kept in mind is that cells containing carbon and other vital stains seem in no way to be hindered from performing their vital functions; they can wander freely, divide by mitosis when almost filled with these pigments, and phagocytose other substances, such as fat, cellular debris, or microorganisms. Fig. 1 shows such a cell in mitosis, with large granules of carbon and Niagara blue grouped at both poles. Such cell division explains the rapid dispersal of carbon particles through foci of active cell proliferation, each successive division resulting in a corresponding diminution in the number of pigment granules per cell.

In order to answer some of the objections that could be raised against deductions based upon the study of fixed and stained tissues, the behavior of the ink particles has been observed *in vivo* in two widely separated groups of animals, the frog and the rabbit.

*Action of Ink in Vivo.*—0.2 cc. of Higgins' ink and distilled water in equal parts, injected into the heart of a pithed frog, can be found circulating in a free state in the capillaries of the mesentery and toe-web for about an hour. After this it is chiefly contained in cells which, in Giemsa films, prove to be mononuclear leucocytes. (This refers to the distribution of the ink in the mesentery and toe-web only.) These often adhere to the endothelium, particularly at the sharp angles of anastomosing vessels, and sometimes penetrate their walls and appear in the tissues. Little or no ink is taken up by the endothelium so long as the circulation continues moderately brisk.

In the rabbit omentum, however, the ink circulates in a free state for less than an hour, in a urethanized animal injected intravenously with 5 cc. of a 50 per cent mixture of Higgins' ink in distilled water and observed in a warm chamber. After  $\frac{3}{4}$  hour the endothelium is liberally dotted with black granules, even in vessels where the circulation is rapid. During  $2\frac{1}{2}$  hours no free ink was seen to leave the vessels of either species of animal; apparently the pigment was always carried out by cells. The results of these observations on two amphibian subjects and one mammal would indicate that there are marked differences in the physiology of their endothelium, just as there is great dissimilarity in their connective tissue cells. It is unsafe to draw conclusions from observations based upon a study of inflammation in Amphibia and then to attempt to apply them to the same process in Mammalia. More work will be done on this subject and reported later.

*Technique.*

After experiments with various rabbit tissues, the fatty omentum was chosen as the most suitable for the purpose. The results were compared with those obtained in wounds of the ear, abdominal wall, and skull; a discussion of these will have to be deferred for the present, as it would make this article too unwieldy. The fatty omentum contains between its two mesothelial layers little else save fat cells and vessels (Fig. 2); fibrous tissue is so scanty as to be almost negligible and the lymphoid taches laiteuses are not encountered in this fatty portion of the organ. The tissue is, therefore, very simple and any added element is quickly detected.

A series of animals was anesthetized and the omentum looped up by through and through sutures into a bunch about 1 cm. in diameter. Rice's No. 100 white sewing silk was always used. The omentum was then dropped back and the abdomen closed by suture. The aseptic lesions thus obtained were removed, either after death by the intravenous injection of fixative under anesthesia, or at operation under ether, at such intervals as would produce lesions representing periods from 2 hours to 5 weeks (2 hours; 1, 1½, 2, 3, 4, 5, 6, 7, 9, and 12 days; 2, 3, 4, and 5 weeks).

The tissues were fixed either in Helly's or in Zenker's fluid, cut to 5 microns in paraffin and stained with Mayer's aqueous carmalum, Van Gieson's stain, and Mallory's aniline blue and his phosphotungstic acid-hematoxylin stains. In the Van Gieson procedure care was taken to use only freshly prepared Weigert's iron-hematoxylin (the iron chloride solution deteriorates rapidly) and to soak the sections for ½ hour or more in water, before counterstaining with Van Gieson's picric acid-acid fuchsin solution. This brings out the nuclear details and the fibroglia fibrils in a way that is impossible to obtain if the counterstain is applied immediately after the hematoxylin. All the animals were given 10 cc. of 1 per cent Niagara blue<sup>3</sup> in distilled water intraperitoneally daily for three doses and thereafter thrice weekly. They received 50 per cent Higgins' waterproof drawing ink in distilled water intravenously; the injections were begun at the time of operation. Up to 1 week they were given 5 cc. daily, those living longer were injected with 2 to 3 cc. three times a week.

Several of the lesions representing critical periods in the series were produced in duplicate or triplicate, on separate animals; especially the 1, 4, and 5 day lesions. None of them showed evidence of infection except one of the 5 day set, in which there was more exudate than could be explained by trauma alone. This infection was, however, insufficient to cause gross changes.

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<sup>3</sup> The Niagara blue was kindly furnished by Dr. George B. Wislocki, of the Department of Anatomy of Johns Hopkins Medical School.

*Macroscopic Examination.*

Macroscopically the omental lesions were swollen and hemorrhagic up to 3 days, thereafter diminishing in size, but increasing in firmness. Their surface was covered with a light coating of fibrin in most cases; in some, where there was slight capillary oozing, it was encased in a thick, whitish envelope. This was purposely produced, as will be seen later. On section the lesions were like the normal fatty omental tissue, with hemorrhagic areas in the early lesion; in the older specimens they appeared pale and fleshy and finally fibrous.

*Microscopic Examination.*

The description of the microscopic changes will be confined as far as possible to the consideration of the behavior of the endotheliocyte and its derivatives, and to that of fiber formation. The other features of aseptic wound healing are too well known to merit further discussion. As there are two fields of study in the experiment, the lesion in the omentum and that in the immediate vicinity of the stitches, they will be considered separately.

*Changes in the Omental Tissue.*—At first there is the usual hemorrhage and exudation of fluid, fibrin, and polymorphonuclears; the capillary endothelium becomes swollen very early in the process (Fig. 3) but does not show much ink at first, as this was administered at the time of operation when the capillary endothelium was as yet unaffected. Numbers of endotheliocytes surround the smaller vessels, and single cells can be found penetrating their walls, one group migrating through the tissues, another tending to become sessile and to form a network of anastomosing cell processes. These groups will be followed separately. By the 2nd day mitotic figures are abundant in the endothelium of the capillary blood vessels and, to a lesser extent, of the lymphatics; they are also found in the wandering, rounded, or spheroidal cells at some distance from the vascular areas. Some of these cells lie just outside of the capillaries, others at a distance, and many contain from one or two, up to many granules of carbon. Very few mitotic figures are found in sessile spindle cells; there are more division figures in the vascular endothelium and immediately outside of the vessels than elsewhere in the sections. The prolifera-

tive activity of the injured tissue is found chiefly in or near vessels. Lymphocytes and plasma cells appear by the 3rd day (Fig. 4).

At the end of 24 hours the wandering group of endotheliocytes begins to surround the damaged fat cells and form a wall about them; but this is more striking on the 3rd day (Fig. 5), when syncytia, or foreign body giant cells, are formed. These are easily found on the 4th day (Fig. 6). Many of the phagocytes, now grown large enough to be called macrophages, show mitotic figures, and mitoses can be found in the protoplasm of the syncytia, as shown in Fig. 7, taken from a 6 day lesion. In one instance a multiple mitosis is seen in one of these giant cells (Fig. 8), resembling those observed in the true tumor giant cells. There is a monaster at the center, lying at the equator of a diaster (seen slightly diagonally); a second diaster, much smaller, is situated in one corner of the cell. This is the first time that mitotic figures have been observed in the syncytia formed in this series of experiments. Phagocytes also invade the pyramidal spaces between fat cells and multiply freely, some of them becoming sessile and intimately connected with a delicate fibrous network (*cf.* Fig. 6), thus joining the sessile group.

By the 4th day wandering phagocytes begin to appear in increasing numbers near the periphery of the omentum, lying in remnants of fibrin which was deposited during the more acute stages of the inflammation. Niagara blue, in granular form, is now very evident in these cells. Plasma cells and lymphocytes continue to increase in number, but are not vitally stained and do not seem to play a formative part in the process. By the 6th day the injured fat cells are reduced in size and largely replaced by syncytia, and there is a definite zone of vitally stained, vacuolated phagocytes at the periphery of the omental sections. Apparently they have removed much of the fat that was liberated by operative trauma. New capillaries are found branching among the fat cells, where they were formerly few in number. The number of plasma cells and lymphocytes decreases.

By the 9th day phagocytes are found penetrating the surface of the omentum and entering the peritoneal cavity, where they lie in fibrin or coagulum (Fig. 9). They may also enter lymphatic spaces in the tissue and form large, pyramidal plugs of granular, blue-stained macrophages. Their task is apparently finished, they have removed the

debris. Many of them degenerate and die, death being indicated by the vital staining of their nuclei with the Niagara blue. Although the morphology of nucleus and cytoplasm is apparently normal, the color (in carmalum sections) is blue instead of the normal carmine, upon which the blue is superimposed. Niagara blue, like trypan, isamine, or pyrrhol blue, or trypan red, is valuable as an indicator of cell death, for it marks the onset of death before the morphology of the cell has changed beyond the degree shown in degeneration. These cells then disintegrate, liberating their contained ink and Niagara blue not as a group of dispersed granules, but as black or blue globules, often as large as erythrocytes. One or more endotheliocytes often lie near such dead phagocytes and appear to pick up their debris.

The tendency of the endotheliocytes to form fixed tissue is not limited to the first 24 hours of the process under observation. They appear to do this throughout the entire series of lesions, so that new tissue is formed first near the vessels, gradually spreading among the fat cells, or along the denuded surface of the omentum (Figs. 10 and 11). At first cells lie free on this surface, surrounded by strands of fibrin. They gradually send out more and more protoplasmic processes, and, as time goes on, fibrous tissue forms in their neighborhood and is penetrated by new capillaries, which in turn furnish more endotheliocytes. There are normally very few fibroblasts present in this tissue; that some of them proliferate and form new fibrous tissue is, of course, probable, but the impression gained from an intensive study of a great many sections from these lesions is that the new tissue is the result of a migration of cells and subsequent proliferation, rather than of the proliferation of cells already present. This impression is intensified by the presence of carbon in many typical fibroblasts.

The changes seen between the end of the 1st week and that of the 2nd are to be ascribed to absorption and fibrosis. The phagocytes remove much of the fat, leaving in place of the large, pale omental fat cells, areas of much smaller fat vesicles. Between these, fibrous tissue is formed (Fig. 12).

*Stitch Absorption.*—After 1 day the stitches are found surrounded by bands of coarse fibers, apparently collagenous in nature. This could be accounted for by the stitch cutting through several layers of



fat cells and gathering the interstitial fibers that lie between them into one strand of connective tissue, or by a coagulation of some material or substance around the stitch. As these fibers often appear rather granular, and as similar fibers, to be discussed later, appear in areas of hemorrhage, the latter explanation seems the better. The only cells present at this time are erythrocytes and polymorphonuclears, which lie between the silk fibers in a coarse, granular coagulum that also gives the color reaction of collagen, though faintly (Fig. 13).

Endotheliocytes invade spaces between the silk fibers on the 4th day; many of them contain ink and show fibroglia fibers. Were it not for the ink, they would pass for fibroblasts from the surrounding connective tissue; but it must be remembered that the surrounding connective tissue is very scanty, while these cells are quite numerous. They next crowd together and form syncytia (Fig. 14), which are well developed by the 6th or 7th day. Among these, numerous discrete cells can be seen, with and without ink granules, and numerous fibroglia and collagen fibrillæ of a delicate, well defined type can be demonstrated. At the same time the coarse, fibrous band surrounding the stitch as a whole is invaded by wandering cells and teased out into a dense network of delicate collagen fibrillæ. This is the first evidence of cellular activity on this particular band of fibers. Some of the cells that penetrate it show ink granules.

By 1 week the syncytia in the stitch have become very large and surround the silk fibers with sheaths of protoplasm, within which lie fibroglia fibrils, ink granules, and Niagara blue. A few days later some of the elongated syncytia begin to show peculiar fringes of very coarse fibroglia fibers, which project from their narrow extremities (Fig. 15). These fibers stain blue with Mallory's phosphotungstic acid-hematoxylin, red with his aniline blue connective tissue stain, and faintly gray with Van Gieson's stain. After 3 to 5 weeks much of the silk is disintegrated and the syncytia are filled with the fragments (Fig. 16). Collagen fibers run in all directions between the remaining silk fibers, and fibroglia binds the cellular elements closely together. The silk is sometimes completely absorbed, and the syncytia, their work completed, break up into masses of epithelioid cells (Fig. 17) after their nuclei have rearranged themselves. Cell boundaries appear between the latter, where none existed in the earlier



lesions. The coarse fibroglia fibers disappear. In place of the fields of syncytia, there are masses of more or less closely packed cells and small syncytia, with few instead of many nuclei.<sup>4</sup> Capillary vessels grow into these areas and are surrounded by numerous endotheliosytes. Ink particles are retained throughout this process by the cytoplasm of the syncytia and the cells into which they resolve. Coarse collagen fibers are interwoven with these cells (Fig. 18).

*Fiber Formation.*—In contrast to normal omentum, in which there is so little fibrous tissue as to be almost negligible (*cf.* Fig. 2), fibers are found soon after inflammation sets in. Their formation is difficult to follow step by step. They are scattered through small masses of fibrin in distended tissue spaces; coarser and more undulating than fibrin threads, they lack the nodal thickenings of these and give a faint collagen reaction. The fibrin is partly removed, partly consolidated into coarser bands, like the torn shreds of a spider-web (Fig. 19) and, by the 4th or 5th day, it is abruptly replaced by a delicate felting of crinkled collagen fibrils (Fig. 20).

It is difficult to interpret the part played by cells in the production of this felting; that the polymorphonuclears remove, or dissolve much of the fibrin seems evident. Sometimes there is a moderate number of spindle cells in such an area, sometimes but two or three (Fig. 21). They may or may not contain ink and are always surrounded by a delicate, cocoon- or sheath-like network of fine collagen fibrillæ; but as fibrils of the same type, or coarser, may be found at a distance from any cells, these sheaths might represent a local reaction to the cells on the part of the intercellular substance, or a product of the cells themselves. The theory that these filaments are formed either from rearranged fibrin, or by a filamentous coagulation of tissue fluids, to be impregnated later with collagen, should be reconsidered. Coarse bands of collagen fibers appear where none are found in control sections, without any obvious association with fibroblasts. Furthermore, they appear near masses of fibrin (Fig. 22) or in vascular areas (Fig. 23). Fig. 22 shows a mass of coarse, almost hyaline fibers that formed beneath a hemorrhagic effusion purposely produced

<sup>4</sup> This breaking up of giant cells has been observed *in vivo* in tissue cultures of bone marrow, in which they form on cotton threads, or the cover-slip itself.

over the omental surface, which was of 4 days standing. It will be seen that there are practically no fibroblasts near these fibers. The latter stain red in Van Gieson sections, blue with Mallory's aniline blue method, and brownish red with phosphotungstic acid-hematoxylin; either they are collagen fibers, or the three best known collagen stains are worthless as such. Similar fibers are found in areas where there has been infarction due to strangulation by the stitches. They do not appear in clots formed outside of the tissue in the peritoneum, fibrin alone apparently does not produce them. Some fluid, either secreted by living cells, or released from them by crushing, coming in contact with another fluid in the clot might combine with it to form a fibrous coagulum, after the manner of a chemical reaction. The resulting fibers would then become impregnated with collagen, or might in themselves represent a collagenous precipitate. Judging from their gradually becoming more and more intense in color reactions the first hypothesis would seem more probable.

That fibroblasts alone do not form fibers of this type is to be inferred from the fact that they invade areas of hemorrhage and grow luxuriantly, forming a dense network of anastomosing cells (Fig. 24). In these masses there are no collagen fibers whatever, and it is also significant that there is little or no fibrin present here. As far as fibroglia fibrils are concerned, there is nothing in this experiment to alter existing theories; that they apparently appear and disappear with readiness is shown in two instances: when fibroblasts undergo mitotic division there is no trace of fibroglia in their cytoplasm, and when syncytia that have apparently contained heavy fibroglia fibers break up into smaller cells, they leave no trace of these fibers as such. It is of some interest that fibroglia is readily demonstrable in these syncytia, which are generally admitted to be of endothelial origin, but in which heretofore only reticulum has been demonstrated by other writers.

The literature on fiber formation is too voluminous to be extensively quoted here; ample discussion and references can be found in any standard text-book on histology or pathology. What has been said here bears out the findings of Baitsell (1915, 1915-16, 1916) to a striking degree, although his papers had not been read at the time of writing this section. While he was unable to obtain a successful red staining

reaction, using Van Gieson's stain, with the collagen fibers he described, and although they stained blue with Mallory's aniline blue method, it will be seen that in the case of the rabbit tissue now under discussion not only these two stains, but also phosphotungstic acid-hematoxylin gave a successful collagen reaction. For a résumé of the literature on this immediate topic the reader is referred to an article by Lewis (1917).

### *Mesothelial Reactions.*

There is very little to say concerning the reaction of the mesothelial covering cells of the omentum; they become detached very early in the process of inflammation and regenerate during the first 3 or 4 days. Areas of local proliferation are found, the cells tending to form small clumps, or heaps, on the denuded surface. The peritoneum thus formed is very readily detached during the process of fixation, particularly over areas where there has been extensive scar formation, so that they appear to be completely denuded. A few cells of the mesothelial type may be found here and there, often partially detached, indicating that the covering layer has been rubbed off by manipulation. The vital staining of the mesothelium is peculiarly scanty; although bathed in a large amount of aqueous 1 per cent Niagara blue, most of this appears to go through the cells without leaving very noticeable traces beyond a few, faintly blue granules. The phagocytes immediately beneath the peritoneum, however, are deeply pigmented both with blue and black granules. This is true of the normal as well as of the injured portions of the omentum after fixation with Helly's or Zenker's fluid.

### DISCUSSION.

For the sake of convenience the endothelial cells under discussion may be considered as belonging to three types, or groups; but it must be understood that these groups are quite artificial. The endothelial cells of the end capillaries appear to retain non-specific or embryonal, characteristics, or to acquire them under the influence of injury; they can then take on one of three types of growth, each of which passes readily into one of the others.

*Group A.*—These behave exactly like those cells observed in the first paper of this series (Foot, 1919), on the reactions to agar injections. Arising from the capillary endothelium, they leave the vessels and wander about in the tissues as phagocytes, and may persist as such to the end of the experiment. They are capable of further proliferation in the tissues, of forming syncytia, which are probably temporary in character, by fusion with one another and by further multiplication of their nuclei after this fusion (*cf.* Figs. 7 and 8). They can remove debris of various sorts, either by digesting it, or by rendering it inert by surrounding it with an "insulation" of protoplasm. They either migrate to the peritoneal surface or enter lymphatics, at the termination of the process that calls them out.

*Group B.*—These cells do not function as phagocytes after leaving the vessels, but become sessile almost immediately and produce new tissue. They differ from fibroblasts in no respect, excepting that they contain ink, and they exhibit all of the characteristic functions of that cell. It is probable that these two groups are freely interchangeable, phagocytes (Group A) becoming sessile, and fixed cells (Group B) rounding up and becoming free phagocytes.

*Group C.*—This group remains specific in as far as the cells of which it is composed are concerned with the production of new capillaries by budding. The endothelium of these new capillaries takes up ink with the same avidity as does that of its parent vessel, provided that the ink be supplied frequently enough throughout the experiment. Its component cells may then undergo the changes which characterize the preceding groups.

Injury or inflammation causes dilatation of the end capillaries, stagnation of the blood stream, and swelling and proliferation of the essentially unstable or non-specific endothelial cells. These can be found in the act of penetrating the vessel walls and leaving the capillaries, each of which becomes surrounded by a distinct zone, or collection of spheroidal cells that are not present in this situation in controls. Many of these cells can be found in mitosis and numbers of them contain ink globules. Despite this proliferation, new vessels are not formed to any extent before the 5th or 6th day of the process. The distribution as shown by a count of 100 unmistakable monasters and diasters, in sections from 5 day lesions and three counts of 50 from 2, 3, and 6 day lesions, is presented in Table I.

The second column includes mitoses found in cells *in situ* in the vascular endothelium; the third, those in cells in the immediate neighborhood of the vessels; the fourth, mitoses found in cells not apparently near any vessel; the fifth, peritoneal cells; and the sixth, cells of spindle shape in various situations. The count of 100 mitoses was made by two observers, to avoid personal error. The variations in the count from day to day are not very marked, if we except that of the 6th, which shows a marked increase in perivascular figures and a diminution in those found in spindle cells. From the average percentages it is clear that 59 per cent of the total number of mitotic figures are in or near the capillaries, 31 + per cent in cells that are at a distance from vascular areas, these cells being rounded and

TABLE I.

Day.	In vascular endothelium.	In immediate proximity to vessels.	At some distance from vessels.	Mesothelial.	In spindle cells.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
2nd.....	44	18	24	6	8
3rd.....	40	16	34	6	4
5th.....	32	19	37	0	12
6th.....	36	30	32	0	2
Average.....	38	21	31.7	3	6.5

some of them containing ink, so that they might well be migrated endotheliocytes; only 3 per cent mesothelial cells, or, more properly, 6 per cent, seen in the younger lesions only; and but 6 + per cent in cells which, from their morphology alone, would be classed as fibroblasts. As previously stated, some of these, too, contain ink.

From the preceding paragraph it will be plain why many of these cells are considered to be endothelial in origin. Aside from the presence of ink there are (a) the predominance of intravascular and perivascular proliferation without a corresponding production of new vessels; and (b) the presence of many new cells of a rounded form, with vesicular nucleus and generous cytoplasm in places where none are found under normal conditions, together with insufficient evidence of proliferation in the preexisting, fixed tissue elements in these localities.

It is said that the perivascular collections of cells arise from the proliferation of fibroblasts closely applied to the outside of the capillaries and not readily visible under normal conditions. It is admitted that if these become detached and rounded up they cannot be distinguished from endotheliocytes, until they again become spindle-shaped and form fibrils. How does the ink get into their cytoplasm? Since it does not pass the vessel wall as such, it must be transferred from cell to cell, which, in this case, would merely fix a foreign body in the tissue, instead of leaving it in a cell that might migrate to the lymphatics and remove it as in anthracosis. In short, we must assume a cell that is so scarce as to be barely demonstrable; we must have it proliferate enormously *in situ*, which cannot be demonstrated in this experiment; and we must have inert particles of material transferred from cell to cell; all this before the theory that these are all connective tissue cells can be maintained. Is it not more probable that the cell becomes laden with ink while it is a part of the vessel (demonstrable *in vivo*) where there is plenty of that substance, and migrates from the vessel, retaining this ink? That these cells can form fibrils of reticulum is well known (Corner, 1920); that the reticulum undergoes transformation into fibroglia and is associated with the production of collagen fibers around the liver sinusoids has also been shown (Kon, 1907-08, Rössle and Yoshida, 1909). In fact, fibrils can be demonstrated readily in the capillary endothelium with Mallory's aniline blue connective tissue stain (red) and with his phosphotungstic acid-hematoxylin method (blue); the differences in color observed in fibers stained by the Bielschowsky-Maresch method may be attributed largely to their size and density, rather than to definitely specific differences.

#### SUMMARY.

The following hypothesis may be deduced from the evidence just submitted: The endothelium of the end capillaries, the fibroblast, and the immediate derivatives of these two are all mesenchymal in origin. Injury produces a reaction wherein there is stasis and hemorrhage, with the exudation of cells and fluids that contain some element or elements that affect these mesenchymal derivatives in such a way as to reestablish their embryonal characteristics. As a result, they form

a local, temporary mesenchyma at the site of injury, proliferate, migrate, and differentiate according to the needs of the case until the injury is repaired. They may form connective tissue, phagocytes, polyblasts, new vessels, etc. Phagocytosis and absorption gradually withdraw the exciting agents from the seat of injury, the temporary mesenchyma returns to adult type, and all that remains is the products of the process that are necessary for scar formation. It is apparently erroneous to think of the mononuclear cells of young granulation tissue as fibroblasts, endotheliocytes, etc., they should be considered as representing various stages in the differentiation of a local mesenchyma until the process of healing is completed, when they return to the adult types represented by these more specific names. The term polyblast, originated by Maximow, is particularly applicable to these cells, for under the conditions they are truly polyblastic; but that they are derived from lymphocytes is not indicated by results of this experiment—the lymphocytes and plasma cells appear to play a part that is unconnected with the formation of new tissue.

No definite statement can be made as to the nature of the stimulating substance, or substances, that bring about this reversion to embryonal type. As indicated, they are associated in some way with the blood, for the most marked cellular metaplasia is noted in connection with areas of hemorrhage, or in proximity to vascular areas. That the exciting substances are contained in the erythrocytes is furthermore indicated by the fact that the most metaplastic areas are found where the mesenchymoid cells are growing among masses of red corpuscles, with little visible fluid or fibrin present (*cf.* Fig. 24). Whether these substances are enzymes or not is purely problematical.

There is very little, in the several hundred sections studied for this and later papers, to indicate that wound healing is an orderly procession of specific cells to designated positions in these wounds. The only tissues retaining their continuity in growth to a degree in any way comparable with the descriptions in some text-books, are the surface epithelium and the vascular buds, or branches. The majority of mononuclear cells of mesenchymal type appear to fill in the injured area by migration, rather than by growth in continuity. Once they have reached a certain point, they react in the various ways just described. There is, of course, a migration of differentiated cells,

such as macrophages, polymorphonuclears, lymphocytes, etc., from the healthy tissue; but the framework, or structural foundation, of the granulation tissue appears to be composed of the practically undifferentiated derivatives of the connective tissue and capillary endothelium. In other words, it is conceivable that cells originating from connective tissue might become phagocytes and *vice versa*, both types temporarily losing all specificity under the influence of some chemical or enzymatic excitant in the exudate. Until we understand the physiology and chemistry that determine the developmental and retrograde changes of the normal, embryonal mesenchyma, as well as those of this temporary mesenchyma, we can never fully comprehend wound healing.

#### CONCLUSIONS.

1. The proliferation of the capillary endothelium of rabbit omentum, in aseptic inflammation, may result in the production (*a*) of new vessels, (*b*) of phagocytic endotheliocytes, and (*c*) of cells which appear to take on the functions of, and to be indistinguishable from fibroblasts.

2. The last two of the above types may be identified by their selective action for colloidal carbon, taken up while they are part of the vessel wall and apparently retained by them throughout the experiment.

3. The three types appear to be freely interchangeable and not distinctly specific groups.

4. Cellular proliferation is almost equally abundant in or near vessels and in free cells at some distance from them, but mitoses in fixed tissue cells constitute only 6.5 per cent of the average total count.

5. Carbon contained in the endotheliocyte does not affect its vital functions appreciably, particularly that of mitotic division.

6. Mitoses may be found, in this experiment, in syncytia (foreign body giant cells); sometimes multiple mitoses, such as occur in tumors, are found in true giant cells among the syncytia.

7. In this experiment collagen fibers are formed, apparently independently of cellular activity, from fibrin or some substance associated with fibrinous clots. The mechanism of this phenomenon is not yet evident.



8. Coarse fibroglia fibers may be formed within the cytoplasm of foreign body syncytia, which are of endothelial origin.

9. The reaction of the omental mesothelium, under the conditions of this experiment, is similar to that of the epithelium in skin wounds; aside from covering denuded surfaces it apparently takes no further part in the process of scar formation.

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#### EXPLANATION OF PLATES.

Figs. 9, 11, 14, 15, 16, 17, and 23 were prepared with Mallory's aniline blue connective tissue stain, the rest are from slides stained by the Van Gieson method. This explains the pallor of the silk fibers in Fig. 13. Wratten filters G and H were used on all Van Gieson photomicrographs, filters B and E used on most of the aniline blue connective tissue stain photographs.

#### PLATE 51.

FIG. 1. Mitosis in spindle cell containing carbon and Niagara blue. Note condition of endothelium in neighboring capillary and distribution of ink. 3rd day.  $\times$  about 780.

FIG. 2. Normal fatty omentum for control purposes.  $\times$  about 390.

FIG. 3. Perivascular proliferation at end of 1st day.  $\times$  about 390.

FIG. 4. Distribution of carbon and plasma cells in upper right corner. 3 days.  $\times$  about 390.

FIG. 5. Endotheliocytes surrounding fat cells. Note ink and diminution in size of fat cells. 3rd day.  $\times$  about 390.

FIG. 6. Same on 4th day. Syncytia and fibers forming.  $\times$  about 390.

## PLATE 52.

FIG. 7. Same after 6 days. Mitosis in syncytium at left. Further diminution in size of fat cells.  $\times$  about 390.

FIG. 8. Multiple mitosis in giant cell at edge of fat. (The black structures are chromosomes and not carbon.) 6th day.  $\times$  about 780.

FIG. 9. Vitrally stained cells in exudate on surface of omentum. 9th day.  $\times$  about 390.

FIG. 10. Fibroblasts containing ink. 6th day.  $\times$  about 390.

FIG. 11. Organization on surface of omentum. 9th day.  $\times$  about 390.

FIG. 12. Fibrosis and great reduction in size of fat cells. 9 days.  $\times$  about 390.

## PLATE 53.

FIG. 13. Silk suture after 1 day.  $\times$  about 390.

FIG. 14. Same after 5 days.  $\times$  about 390.

FIG. 15. Same after 12 days. Note coarse fibroglia fibers.  $\times$  about 390.

FIG. 16. Same after 3 weeks. Silk fibers becoming striated and less opaque.  $\times$  about 390.

FIG. 17. Same after 35 days. Epithelioid cells developing from syncytia as these break up; silk almost absorbed.  $\times$  about 390.

FIG. 18. Silk absorbed; collagen fibers forming at site of syncytia which are breaking up.  $\times$  about 390.

## PLATE 54.

FIG. 19. Clot after 2 days. Note wavy fibers that appear and compare them with the fibrin.  $\times$  about 390.

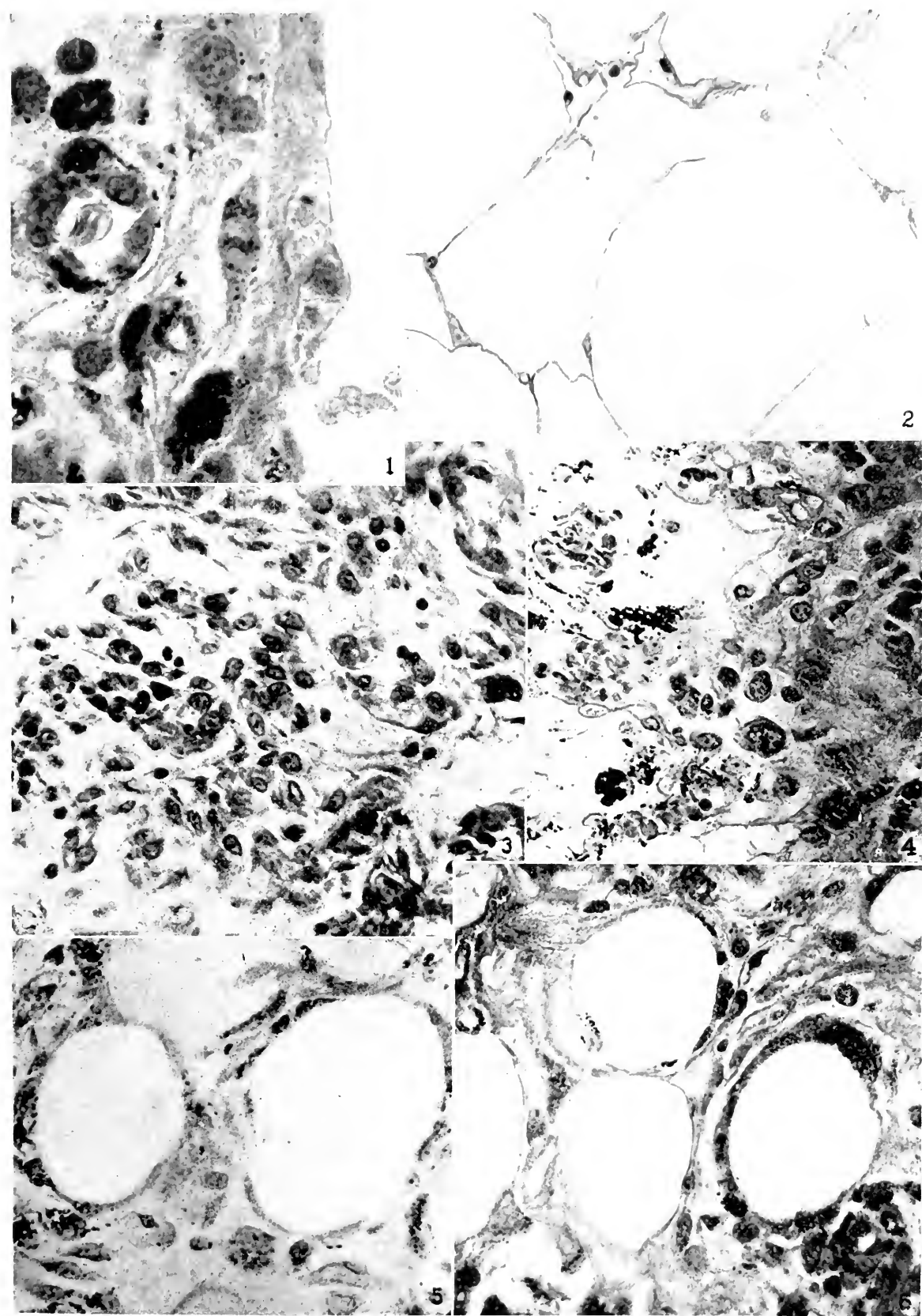
FIG. 20. Fibrin absorbed; new collagen fibers appearing. 6th day.  $\times$  about 390.

FIG. 21. Coarse fibers in clot, two or three fibroblasts among them. 3rd day.  $\times$  about 390.

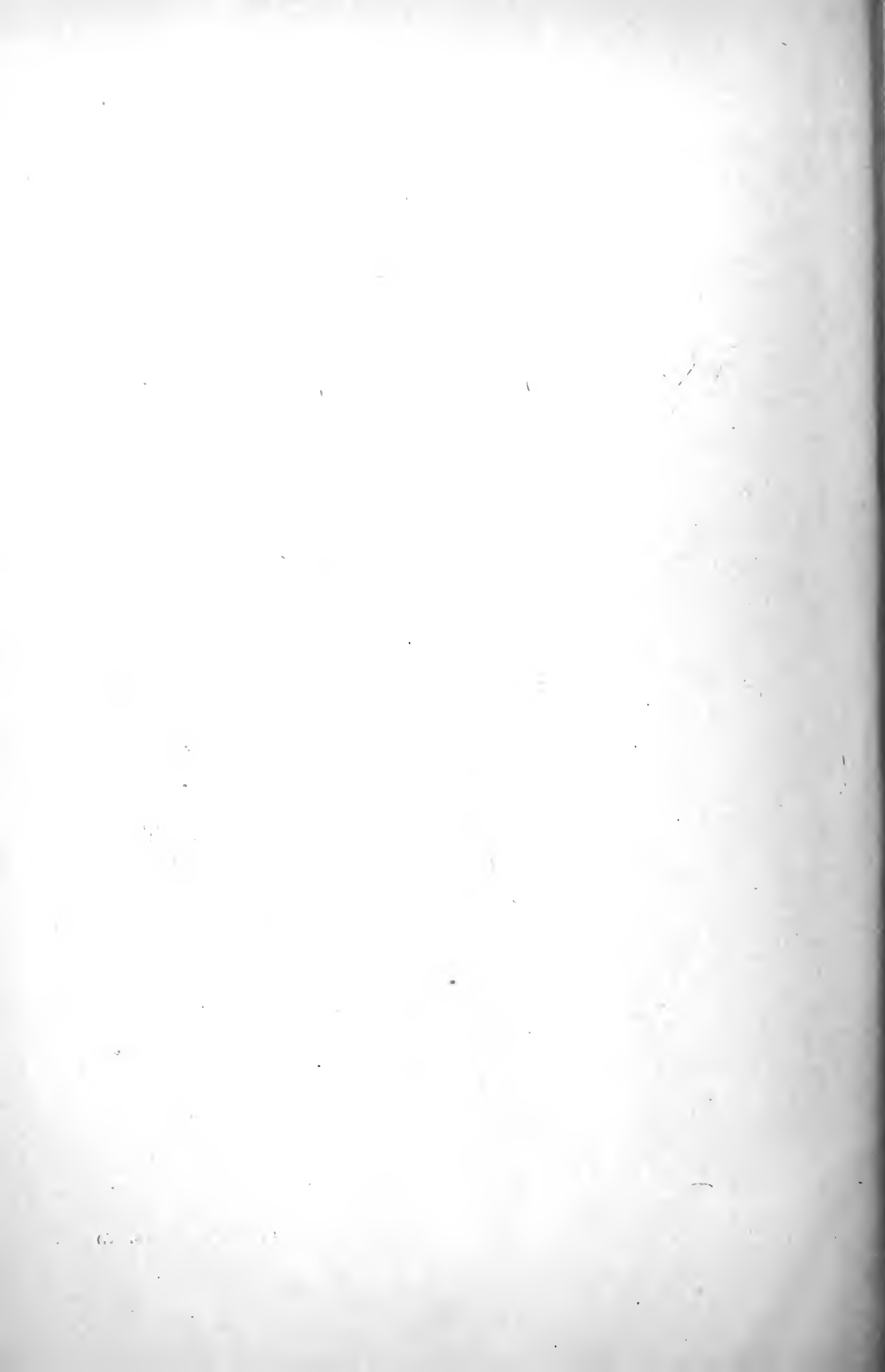
FIG. 22. Coarse collagen fibers formed between exudate and fibrin in tissue. Note comparative absence of fibroblasts. 4th day.  $\times$  about 390.

FIG. 23. Fibers in vascular area; cell leaving neighborhood of capillary just below center. 6 days.  $\times$  about 390.

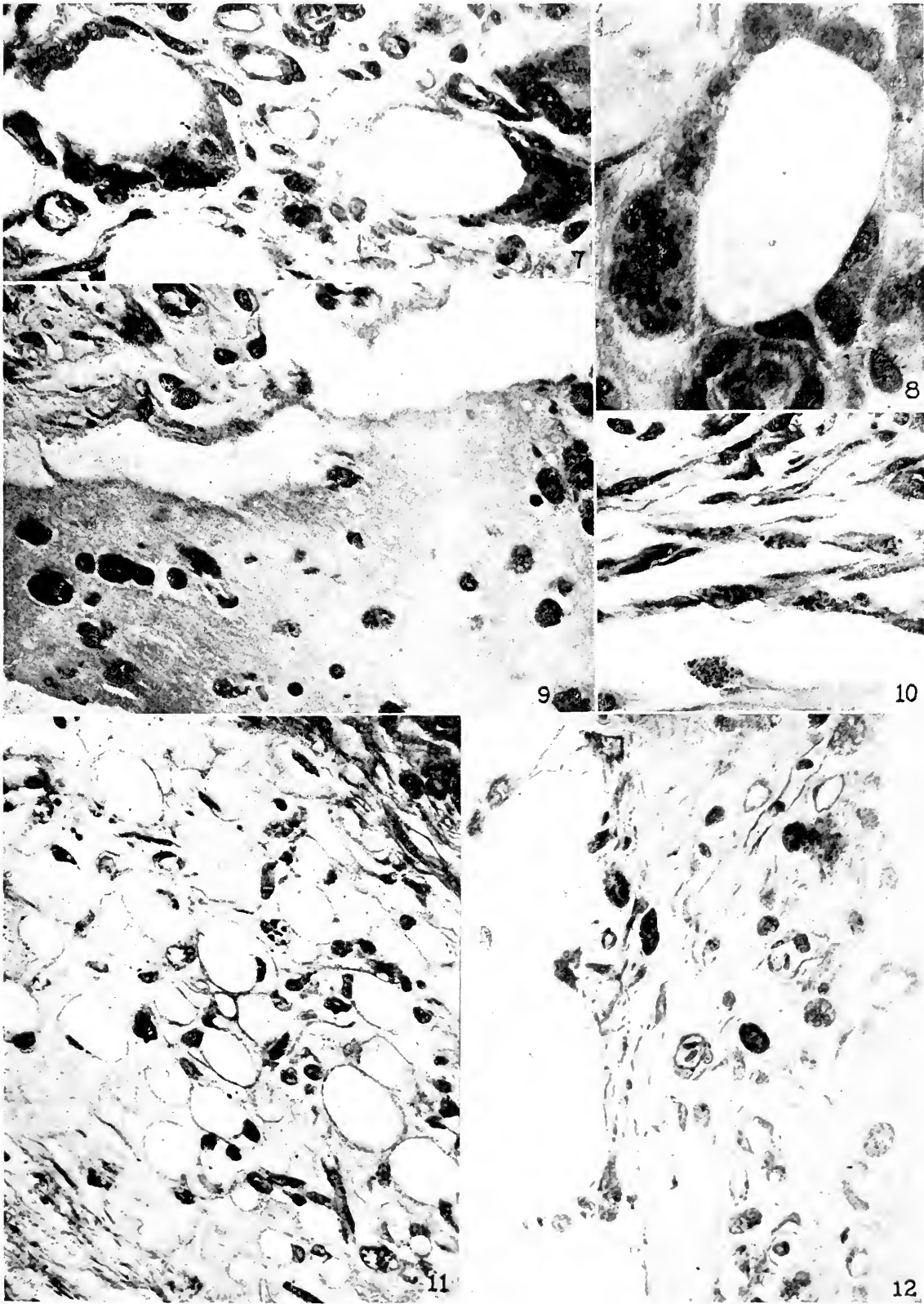
FIG. 24. Metaplastic fibroblasts growing in a fibrinous hemorrhage.  $\times$  about 390.



(Foot: Endothelial reactions. V.)



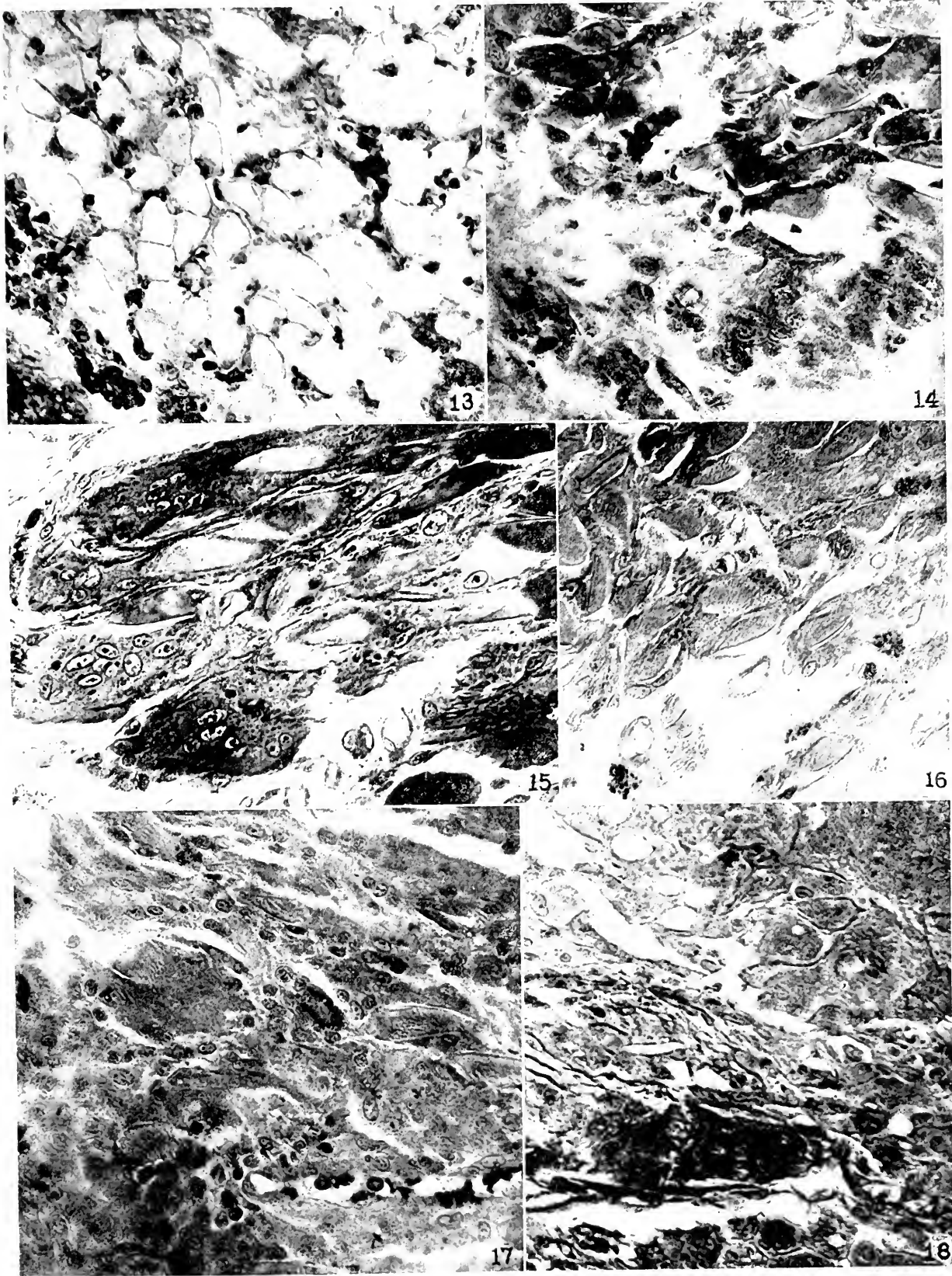
562



(Foot: Endothelial reactions. V.)



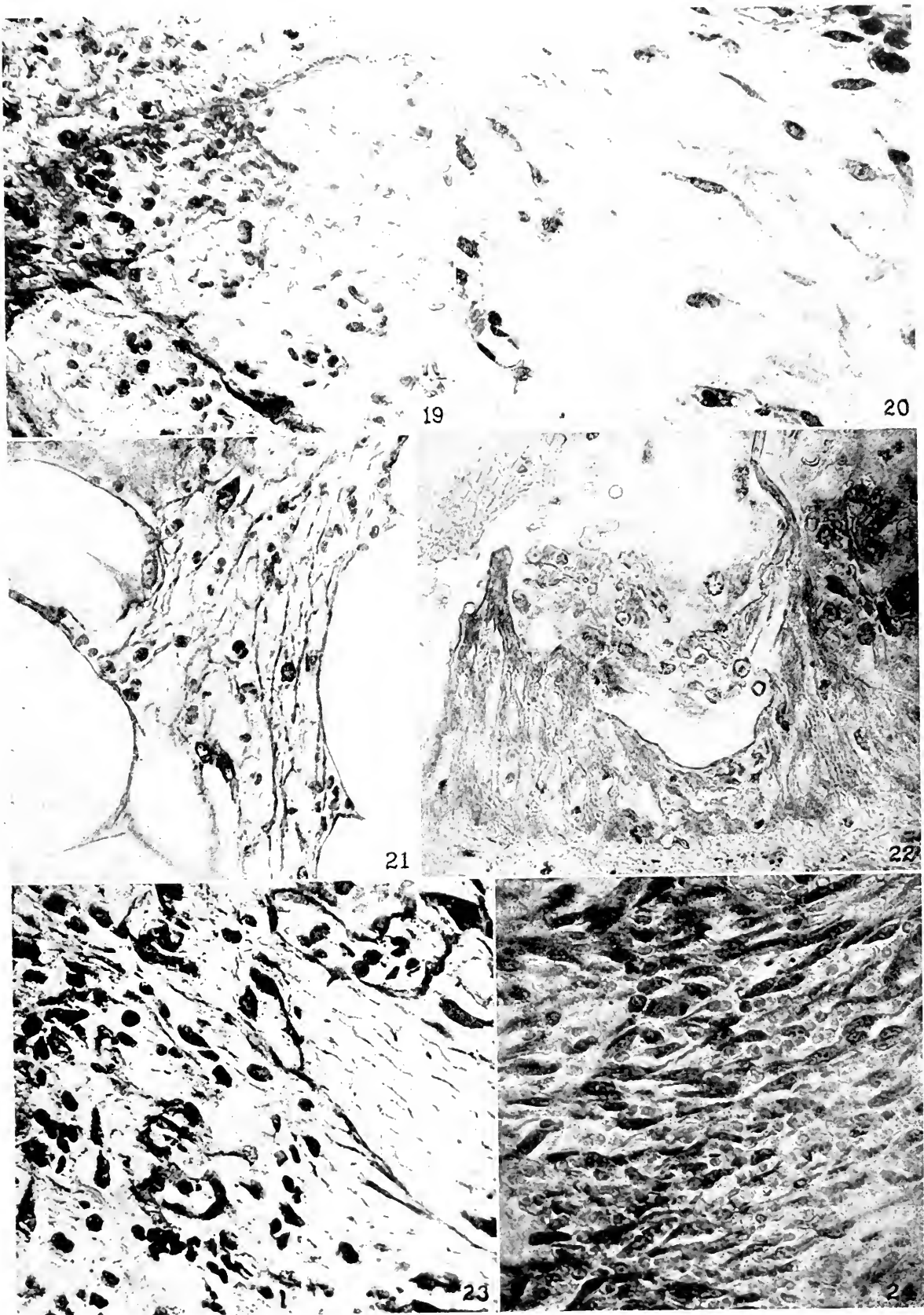




(Foot. Endothelial reactions. V.)







(Foot: Endothelial reactions. V.)



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